Industrial Pharmacy
Faculty of Pharmacy
University of Helsinki
Finland

Comparison of disaccharides and polyalcohols as stabilizers in freeze-dried protein formulations

by

Petteri Heljo

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Pharmacy of the University of Helsinki, for public examination in Auditorium 1041 at Biocenter 2 (Viikinkaari 5E, Helsinki) on March 1st 2013, at 12.00 noon.

Helsinki 2013

Supervisors Professor Anne M. Juppo
Industrial Pharmacy
Faculty of Pharmacy
University of Helsinki
Finland

Docent Kirsi Jouppila
Department of Food and Environmental Sciences
Faculty of Agriculture and Forestry
University of Helsinki
Finland

Reviewers Professor Thomas Rades
Department of Pharmacy
University of Copenhagen
Denmark

PD Dr. Hanns-Christian Mahler
Pharmaceutical Development Biologics Europe
F. Hoffmann-La Roche Ltd.
Switzerland

Opponent Professor Robert T. Forbes
Bradford School of Pharmacy
School of Life Sciences
University of Bradford
United Kingdom

ISBN 978-952-10-8616-8 (Paperback)
ISBN 978-952-10-8617-5 (PDF)
ISSN 1799-7372

Helsinki University Printing House
Helsinki 2013

Cover: Adaptation of a scanning electron microscope image depicting freeze-dried melibiose (magnified 10 000 times).

Abstract

Heljo VP., 2013. Comparison of disaccharides and polyalcohols as stabilizers in freeze-dried protein formulations

Dissertationes Biocentri Viikki Universitatis Helsingiensis, 4/2013, pp. 64
ISBN 978-952-10-8616-8 (Paperback), ISBN 978-952-10-8617-5 (PDF, http://ethesis.helsinki.fi), ISSN 1799-7372

Protein-structured pharmaceuticals have become very important for treating several chronic and acute illnesses, but their commercialization is sometimes impeded by their poor stability in aqueous solutions. In some cases, protein stability may be better in dry than in aqueous formulations, and freeze drying (lyophilization) is the most commonly used method for manufacturing such products. However, excipients are needed to keep proteins from degrading during processing and storage, and the current selection of stabilizing compounds accepted by the regulatory authorities is limited. The aim of this thesis was to compare the stabilizing efficacies of different disaccharides and polyalcohols in protein formulations.

Firstly, the efficacies of 11 disaccharides and polyalcohols as β-galactosidase-protecting compounds were characterized at three different concentrations. Disaccharides were generally more effective than polyalcohols, which appeared to be caused in some cases by their lower crystallization tendency. Four disaccharides (cellobiose, melibiose, sucrose and trehalose) were selected for further secondary structure stabilization studies, which corroborated the results of the activity analysis studies that the protein-protecting efficacies of trehalose and melibiose differed from those of sucrose and cellobiose.

Secondly, the water plasticization and crystallization properties of the four disaccharides were studied after freeze drying by storing them at different relative humidity atmospheres. Cellobiose and sucrose were shown to crystallize faster and at lower relative humidity than trehalose and melibiose. The water plasticization properties of sucrose were shown to differ from the other disaccharides. Melibiose was most stable of the four compounds in amorphous state, which may have partly been caused by its slower molecular relaxation rate compared to trehalose.

Thirdly, the effect of freeze drying parameters on polyclonal IgG stability was studied in order to find out how much the stability of such antibodies could be affected by processing conditions in formulations containing trehalose. While varying the primary drying pressure and secondary drying heating rate did not directly affect protein stability, increased aggregation did occur at lower primary drying pressures when sodium phosphate buffer was used in the formulation. This may have been caused by buffer freeze-concentration, which may have been more significant when the sample temperature was lowered below the glass transition temperature of the freeze-concentrated phase. Sodium chloride was also shown to reduce the Z-average diameter of the rehydrated polyclonal IgG lyophilizates, which may have been caused by a decrease in protein aggregation.

Finally, the efficacies of trehalose and melibiose in protecting a monoclonal antibody (rituximab) from degradation during freeze drying and storage at different relative humidity atmospheres were compared by storing the lyophilizates up to 3 months. Melibiose was generally more effective than trehalose in inhibiting secondary structure alterations, fragmentation and aggregation during drying and storage. Storage at dry conditions (RH 5%) was shown to be more harmful for rituximab in the lyophilized formulations than storage at more humid conditions (RH 11-23%), which may have been caused by the susceptibility of the protein to drying-induced degradation.

Acknowledgements

This study was carried out at the Division of Pharmaceutical Technology, Faculty of Pharmacy, University of Helsinki (UH, Finland) and at the Division of Drug Delivery Technology, Leiden/Amsterdam Center for Drug Research, Leiden University (LU, The Netherlands) during the years 2007 – 2012. I wish to express my deepest gratitude for the people involved in this work.

First and foremost, I would like to thank my supervisors, Professor Anne Juppo (UH) and Docent Kirsi Jouppila (UH), for their never ending support and help during these years. Especially Anne is acknowledged for pushing me forward and infusing me with hope in times of doubt. If it were not for you, I would not have had the guts to aim this high. Also, this work would not have been possible without the collaboration and expertise of Professor Wim Jiskoot (LU), who was kind enough to welcome me to his laboratory and to offer his assistance and expertise whenever I needed it (which, incidentally, was often).

I would also like thank Doctors Vasco Filipe (LU), Mikko Tenho (Univerity of Turku, UT) and Gebregenus Yohannes (UH) for carrying out vital measurements, as well as teaching me the theoretical aspects and practicalities of different analytical techniques. Your participation gave many parts of this work its scientific backbone. Also, I cannot overestimate the importance of the work done by Antti Nordberg (UH) and Helena Harju (UH), which not only resulted in two excellent Master's theses, but also helped me in completing this work. Furthermore, Tommi Virtanen (UH), Timo Hatanpää (UH) and Stefan Romeijn (LU) are gratefully acknowledged for their most significant contributions in many of the experiments that were carried out during the course of this thesis. I would also like to thank Professor Sirkka-Liisa Maunu (UH) and Docent Jarno Salonen (UT) for their help in facilitating the experiments in their respective units, and for their assistance in result interpretation.

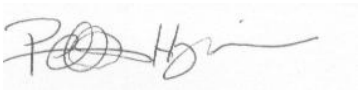
Vetcare Oy played a crucial part in the early stages of this work by providing material funding and exchanging ideas. Especially, I would like to mention the help of Doctors Shea Beasley and Kalevi Heinonen for encouraging me to present my early findings in conferences.

Very significant contributions were also made by Professor Thomas Rades (University of Copenhagen) and Privatdozent Hanns-Christian Mahler (F. Hoffmann-La Roche Ltd.), who reviewed this work and presented their constructive ideas on how to improve it further. I was most pleased to be guided by such esteemed researchers.

I cannot think of better places to work than the Division of Pharmaceutical Technology (UH) and the Division of Drug Delivery Technology (LU). The highlights of my workdays have always been the coffee room chats, barbeque parties and other random events with the people I am proud to call my friends. Thank you for making me laugh, for I have sorely needed that. I would also like to salute the members of *Apteekki Mies* for sticking together despite long distances and the responsibilities of work and family, and all my friends, old and new, for keeping my feet on the ground and reminding me of the important things in life.

Finally, perhaps my deepest thanks go to my parents and brother, Antti, Nina and Samuli, for always being there for me, and to Tiia for your love and understanding. You have constantly infused me with hope. How I can repay you, I may never know.

In Helsinki, on the 5th of February, 2013



Petteri Heljo

Table of contents

Abstract i
Acknowledgements ii
Table of contents iii
List of original publications v
Abbreviations and symbols vi
1 Introduction 1
2 Literature overview 2
2.1 Protein structure and therapeutic proteins 2
2.1.1 Protein structure 2
2.1.2 Therapeutic proteins 3
2.2 Amorphous state and water sorption 4
2.2.1 Structure and different transitions in amorphous state 4
2.2.2 Molecular mobility of amorphous compounds 6
2.2.3 Water sorption and its effect on molecular mobility 7
2.3 Protein instability 8
2.3.1 Factors affecting protein stability in aqueous solutions 9
2.3.2 Factors affecting protein stability in freezing aqueous solutions 10
2.3.3 Factors affecting protein stability in amorphous state 11
2.3.4 Protein aggregation and fragmentation 12
2.3.5 Product-related immunogenicity 14
2.4 Freeze drying 15
2.4.1 Freezing 16
2.4.2 Primary drying 18
2.4.3 Secondary drying 21
2.4.4 Significance of freeze drying as a protein stabilization method 22
2.5 Excipients used in freeze-dried pharmaceutical products 23
2.5.1 Low molecular weight saccharides and polyalcohols 23
2.5.2 Surfactants 25
2.5.3 Salts and buffers 25
2.5.4 The need for new excipients for protein formulations 26
3 Aims of the study 27
4 Experimental 28
4.1 Materials, formulations and protein purification (I-IV) 28
4.2 Methods 29
4.2.1 Freeze drying (I-IV) 29
4.2.2 Sample storage 29
4.2.3 Protein in vitro activity and binding analysis (I, III) 30
4.2.4 Lyophilizate solid state analysis (I-IV) 31
4.2.5 Sample visualization (II) 31
4.2.6 Molecular mobility measurement (II) 32
4.2.7 Protein secondary structure analysis (I, III, IV) 32
4.2.8 Protein aggregation, fragmentation and monomer recovery analysis (III, IV) 32
4.2.9 Multivariate and statistical analysis (I, III) 34

5 Results and discussion 35
5.1 General remarks 35
5.2 Initial excipient comparison and the selection of compounds for further studies (I) 35
5.2.1 The enzymatic activity of β-galactosidase after freeze drying 35
5.2.2 Factors contributing to freeze-dried β-galactosidase stability 36
5.3 The effect of disaccharide physical properties on protein secondary structure retention during storage (I, II) 38
5.3.1 Changes in β-galactosidase secondary structure during storage 38
5.3.2 Water sorption and crystallization kinetics in pure amorphous disaccharides 41
5.3.3 The plasticizing effect of water sorption on the molecular mobility of pure disaccharides 42
5.3.4 Consequences of physical stability differences between excipients 44
5.4 Studying the effect of freeze drying parameters and formulation on IgG stability (III) 45
5.4.1 Varying the processing conditions 45
5.4.2 Varying the formulation composition 48
5.4.3 Factors to be taken into account when freeze drying IgG-class proteins 48
5.5 Comparing the efficacies of trehalose and melibiose as cryo-/lyo-protecting excipients for rituximab during freeze drying and storage (IV) 49
5.5.1 Protein purification and lyophilizate physical properties 49
5.5.2 Effects of freeze drying and storage on rituximab structure and sample particle contents 50
5.5.3 Factors affecting rituximab stability during and after drying 53
5.6 Using melibiose as a freeze drying excipient (I, II, IV) 54
6 Conclusions 56
References 57

List of original publications

This thesis is based on the following publications, which are referred to in the text by their respective roman numerals (I-IV).

I Heljo V.P., Jouppila K., Hatanpää T.T., Juppo A.M., 2011. The use of disaccharides in inhibiting enzymatic activity loss and secondary structure changes in freeze-dried β-galactosidase during storage. Pharm. Res. 28(3): 540-552.

II Heljo V.P., Nordberg A., Tenho M., Virtanen T., Jouppila K., Salonen J., Maunu S.L., Juppo A.M., 2012. The effect of water plasticization on the molecular mobility and crystallization tendency of amorphous disaccharides. Pharm. Res. 29(10): 2684-2697.

III Heljo V.P., Harju H., Hatanpää T.T., Yohannes G., Juppo A.M., 2012. The effect of freeze drying parameters and formulation composition on IgG stability during drying. Submitted.

IV Heljo V.P., Filipe V., Romeijn S., Jiskoot W., Juppo A.M., 2013. Stability of rituximab in freeze dried formulations containing trehalose or melibiose under different relative humidity atmospheres. J. Pharm. Sci. 102(2): 401-414.

Abbreviations and symbols

$\Delta\alpha$	Increment of thermal expansion coefficient at glass transition
ΔTsec	Secondary drying heating rate
Ø	Diameter
ρ	Density
τ	Structural relaxation time of amorphous compound
τ_0	Pre-exponential factor (vibrational lifetime of a molecule)
AF4	Asymmetric flow field flow fractionation
API	Active pharmaceutical ingredient
A.U.	Arbitrary units
a_w	Water activity
BET	Brunauer-Emmett-Teller equation
BSA	Bovine serum albumin
C	Heat constant of surface sorption (in BET)
C_g'	Solute concentration of the maximally freeze-concentrated amorphous phase
C_{crit} (H2O)	Critical water content (%-w/w), where T_g = 25°C
CCF	Face-centered central composite design (in experimental design)
CD	Circular dichroism
CPMAS	Cross polarization magic angle spinning
CSD	Cambridge Structural Database
D	Strength parameter
DLS	Dynamic light scattering
DSC	Differential scanning calorimetry
e.g.	*Exempli gratia* (for example)
ELISA	Enzyme-linked immunosorbent assay
etc.	*Et cetera* (and so on)
FT-IR	Fourier-transform infrared spectroscopy
GI	Gastrointestinal
GT	Gordon-Taylor equation
H	Enthalpy
HP-SEC	High-performance size-exclusion chromatography
H_2O	Water
ICH	International Conference on Harmonization
i.e.	*Id est* (that is)
IgG	Immunoglobulin G
IMC	Isothermal microcalorimetry
INF	Interferon
K	Material coefficient (in GT)
K_{ex}	The exchange coefficient
K_{H2O}	Water binding constant
K_S	Solute binding constant
KF	Karl-Fisher titration
LO	Light obscuration
m	Mass
m_0	Monolayer water content (in BET)
mAb	Monoclonal antibody

mT	Millitorr
MALLS	Multi-angle laser light scattering
MRE	Mean residue ellipticity
MWCO	Molecular weight cutoff
N	Native protein folding
NTA	Nanoparticle tracking analysis
ONPG	o-nitrophenyl-β-D-galactopyranoside
p_w^0	Equilibrium partial vapor pressure of pure liquid water
p_w	Equilibrium partial vapor pressure of water in a system
P	Protein
PC	Principal component (in multivariate analysis)
PDI	Polydispersity index (in DLS analyses)
PEG	Polyethylene glycol
PES	Polyethersulfone
PLS	Partial least squares (in multivariate analysis)
Pprim	Primary drying pressure
PS(20/80)	Polysorbate (20/80)
Q^2	Goodness of prediction value (in PLS models)
R^2	Goodness of fit value (in PLS models)
RH	Relative humidity
s	Solute
S	Entropy
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
ssNMR	Solid state nuclear magnetic resonance
T	Temperature
T_1H	Longitudinal proton relaxation time constant (ssNMR)
T_0	Temperature of zero configurational entropy
T_c	Collapse temperature of amorphous phase
T_d	Protein denaturation temperature
T_{eu}	Eutectic temperature
T_f	Fictive temperature
T_g	Glass transition temperature
T_g'	Glass transition temperature of maximally freeze-concentrated solution
T_m	Melting temperature
T_m'	Onset melting temperature of ice
U	Denaturated protein folding
TFF	Tangential flow filtration
TGA	Thermogravimetric analysis
V_f	Free volume
w	Weight fraction
%-w/w	Concentration percentage (weight per weight)
XRPD	X-ray powder diffractometry
Z-ave	Z-average diameter (in DLS analyses)

1 Introduction

Proteins are inherently unstable molecules. One of the main reasons for this is that compared to small organic molecules, proteins are significantly larger and more complex in structure. When putting this into perspective in the case of drug molecules, it says something about the enormous size of proteins that the mass of rituximab (a monoclonal antibody with M_w = 144 000 g/mol) is roughly 700 times that of the mass of ibuprofen (a common non-steroidal anti-inflammatory drug with M_w = 206 g/mol), whereas the mass of the Earth is "only" about 80 times that of the Moon. While an increase in size does not automatically cause stability issues for different molecules, it does tend to amplify their structural complexity, which in turn may increase the number of relevant degradation pathways.

When using proteins as active pharmaceutical ingredients (APIs), their bioactivity is dependent not only on their primary amino acid sequence, but on their three dimensional structure as well. Structural alterations in the native conformation of pharmaceutical proteins, such as monoclonal antibodies (mAbs), can lead to loss of therapeutic activity and increased immunogenicity (Wang et al., 2010). Therefore, issues with protein stability during processing and storage may impose a significant bottleneck for the commercialization of protein-structured pharmaceuticals (Randolph and Carpenter, 2007). However, there is much commercial interest in the production of stable protein-structured APIs, since they offer significantly improved therapy options compared to small-molecule compounds.

One possible method of increasing the storage stability of a protein formulation is to dry it, but this is not usually the most preferred option. This is because the process is expensive and it may even degrade the product if not done correctly, which means that if a protein is sufficiently stable in aqueous solution, it is not normally dried. However, in some cases a sufficient shelf life (preferably 2-3 years at 2-8°C) cannot be achieved if the protein is formulated as an aqueous solution. Another drawback of aqueous formulations is that they must be stored as refrigerated or frozen, whereas drying can in some cases allow the product to be stored in room temperature. Furthermore, shipping aqueous protein solutions can be detrimental to the product, as shaking subjects proteins to an increased air/water interface due to bubble formation and may result in aggregation (Mahler et al., 2005). Mostly due to these factors, drying the protein formulation is sometimes a justifiable stabilization strategy.

Freeze drying (lyophilization) is the most common method of drying therapeutic proteins, but it requires careful formulation when choosing the correct excipients and designing the drying cycle (Wang, 2000). Still, it may often be the most feasible way of stabilizing certain proteins, so it remains an important, albeit expensive and time consuming, production method for biopharmaceutical drug products. Disaccharides such as trehalose and sucrose are commonly employed as protein-protecting excipients in marketed lyophilized drug products (Wang et al., 2007), but other disaccharides have not been frequently used. With an ever growing number of protein-structured pharmaceuticals in research pipelines, the need for new stabilizing excipients is likely to grow too.

This thesis focuses on improving protein stability during freeze drying and the subsequent storage. Emphasis is placed on comparing the use of disaccharide- and polyalcohol-structured excipients that are already used in biopharmaceutical drug products to those that are not currently included in any marketed products. The work aims to bring insight into protein stabilization mechanisms during processing and storage by first using two nontherapeutic model proteins in the studies. This knowledge will then be put into practice, when an attempt will be made to produce lyophilized formulations that are stable under stressed storage conditions by using a currently marketed mAb as the model protein.

2 Literature overview

2.1 Protein structure and therapeutic proteins

2.1.1 Protein structure

The backbone of human proteins consists of up to 20 different naturally occurring amino acids that are linked together by peptide bonds. Given that a single protein may weigh several hundred kilodaltons, and that other molecules such as carbohydrates may also be attached to the peptide backbone as side chains, there is practically an endless number of protein structures available. The order, or sequence, in which the amino acid are arranged is often referred to as the primary structure of the protein (Branden and Tooze, 1999), and an example of a simple primary structure is shown in Figure 1. What makes the structural diversity of proteins even more complex, however, is that their biological activity is dependent not only on their amino acid sequence, but their three-dimensional structure as well. The primary structure adopts simple energetically favourable three-dimensional forms, known as secondary structures, based on the sequence of amino acids and several kinds of intramolecular bonds such as hydrogen- and covalent bonds (Fig. 1). Common secondary structures include α-helices, β-sheets and –turns, and sometimes a large part of the protein may appear randomly oriented as well. Adjacent secondary structures may carry out simple biological functions, such as acting as binding sites for other molecules. The overall three-dimensional structure of a single polypeptide chain is known as its tertiary structure, and it may possess several active sites with different biological functions (Fig. 2). Furthermore, multimeric proteins that consist of several polypeptide chains are said to possess an additional quaternary structure (Fig. 2), where individual tertiary structure subunits may function independently or cooperatively.

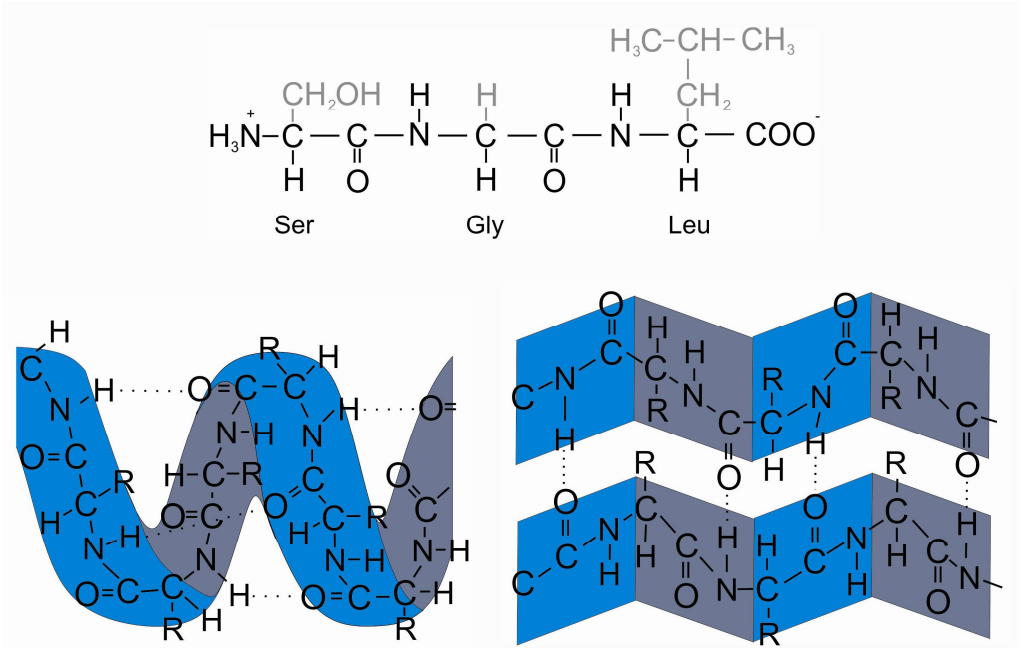


Figure 1. *Above: Primary structure of the tripeptide serylglycylleucine (R-groups marked with gray); Below: α-helix (left) and β-sheet (right) secondary structures, with hydrogen bonds depicted as dotted lines.*

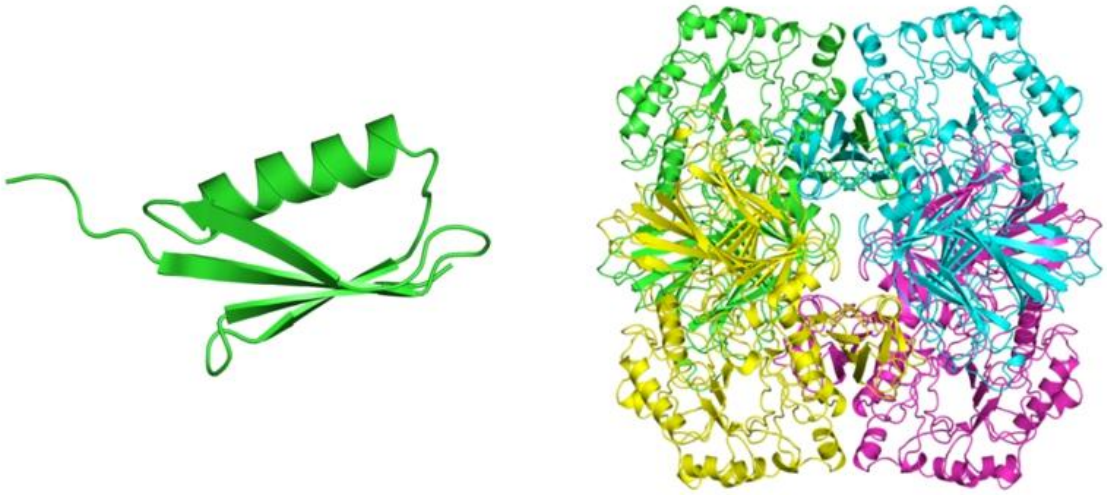


Figure 2. *Left: Tertiary structure of protein G of* Streptococcus sp. G148 *according to Butterworth et al. (1998); Right: Quaternary structure of α-galactosidase sucrose-kinase according to Bruel et al. (2011), where individual polypeptide chains are coloured with different colours. The images have been obtained from The Protein Databank in Europe (PDBe, http://www.ebi.ac.uk/pdbe/).*

The so-called native state of a protein, which represents its folded and biologically most commonly observed structure, is not a rigid unit, but a dynamic system which continuously fluctuates between a limited number of conformations (Jaenicke, 1991). In an aqueous solution, the most energetically favourable conformations are usually formed when the non-polar protein side-chains are directed against each other, forming a hydrophobic core that is surrounded by a polar (hydrophilic) surface (Branden and Tooze, 1999). In addition to the hydrophobic/-philic forces, protein conformation is further stabilized by intramolecular bonds, such as hydrogen bonds and disulphide linkages between different polypeptide chain segments. To cause a change in the three-dimensional conformation, a protein must either be subjected to an energy that exceeds the activation enthalpy of unfolding, or its surroundings must be altered in such a way that the previous native conformation is no longer the energetically most stable one.

2.1.2 Therapeutic proteins

Therapeutic proteins are proteins that can be used to treat or prevent illness in patients. Due to their more complicated three-dimensional structure, they are often differentiated from therapeutic peptides, which are sometimes defined as smaller polypeptide chains comprising of < 50 amino acid residues (Vlieghe et al., 2010). By following this classification, it can be estimated that the number of therapeutic proteins or protein fragments (discounting vaccines) that are or have at one point been marketed in the US, Europe and/or Japan is now over 130. Protein-structured API are used to treat myriad of clinical conditions, ranging from simple gastrointestinal (GI) tract lactase enzyme deficiency (lactose intolerance) to more serious illnesses, such as diabetes, blood clotting disorders and different forms of cancer. A major factor limiting the peroral administration of therapeutic proteins has been their modest permeation through biologic barriers, such as epithelial cell layers, as well as their instability in the presence of the protein-catabolizing enzymes of the GI-tract. Therefore, protein-structured APIs have thus far mostly been administered parenterally. However, pharmaceutical companies have been eager to search for alternative administration routes, especially if the protein in question has a high market value. One such

example is insulin, which has been marketed as a dry powder inhalation (Exubera®, Pfizer Inc., NY, USA, discontinued in October 2007) and has recently been clinically tested as a peroral formulation by several companies (Akkati et al., 2011), such as Biocon Ltd. (Bangalore, India) and Novo Nordisk A/S (Bagsvaerd, Denmark).

One of the most important therapeutic protein groups is certainly antibodies. This is because they can be engineered to bind to specific substrates, as well as conjugated to other therapeutic molecules in order to increase their efficacy and reduce the dose needed to produce a therapeutic effect (Wang et al., 2007). The development of therapeutic antibodies has advanced in leaps ever since Köhler and Milstein (1975) developed a method for producing monoclonal antibodies (binding to only one specific epitope) by *in vitro* cell fusion. The first mAb drug product, Orthoclone OKT3® (Janssen-Cilag Ltd., Buckinghamshire, UK), was approved for market in 1986 (Wang et al., 2007), and in late 2012, 32 mAb drug products had received a marketing authorization (discounting diagnostic products). The therapeutic scope of antibodies is broad, ranging from the treatment of autoimmune diseases such as Crohn's disease and rheumatoid arthritis to cancerous and infectious diseases, as well as transplant rejection therapies. In 2011 five out of the top 20 drug products by sales were mAbs, and the combined global sales of all mAbs on the market were estimated at $44.6 billion, which was approximately 5% of the total global pharmaceutical sales (BCC-Research, 2012). Furthermore, this figure is expected to rise to $58 billion by 2016, which means that mAbs represent a significant source of revenue for the pharmaceutical industry. Currently, 10 out of the 32 mAb drug products that have received a marketing authorization are produced by freeze drying, meaning that it remains a relevant production method for therapeutic antibodies. Since freeze-dried protein drug products are partially or completely amorphous systems, in order to understand them, one must first understand the nature of the amorphous state.

2.2 Amorphous state and water sorption

2.2.1 Structure and different transitions in amorphous state

The atoms or molecules that form solid matter are often depicted as being arranged in crystalline form. Despite exhibiting some irregularities (defects), the crystalline form is a highly ordered state (Fig. 3) and its physicochemical properties are very uniform throughout the crystal lattice. A transition from the crystalline phase to another, such as to liquid, manifests as a stepwise change in enthalpy (H), entropy (S) and free volume (V_f), and it involves a fixed latent heat that is consumed during the phase change without a change in temperature (as shown in Fig. 4). Such a transition is known as a 1st order phase transition. However, not all materials that appear solid by nature are crystalline. Liquids that are cooled below their melting temperature (T_m) do not necessarily crystallize under experimental time scale, but as shown in Figure 4, they may form supercooled liquids instead. Cooling such a liquid further increases its viscosity, and at some point the movement of individual molecules becomes so slow that they no longer have time to rearrange as their most energetically favorable liquid conformation (Ediger et al., 1996). This causes H, S and V_f to deviate from the liquidus curve and the supercooled liquid eventually becomes practically immobile, as molecules can no longer move past each other under experimental time scale (i.e. their translational molecular mobility becomes very limited, but significant rotational and vibrational mobility is still retained). The sudden change in viscosity is often referred to as

the glass transition (T_g, α-transition), and while the glassy material below T_g may appear totally solid, its molecular lattice exhibits only short range order (as seen in Fig. 3). Therefore, the glassy state is differentiated from both the crystalline and liquid states, since on molecular level its structure is similar to a highly viscous liquid, but its specific heat capacity and thermal expansion coefficient are closer to crystalline than liquid form (Kauzmann, 1948). Despite this, glasses are still often considered as solids.

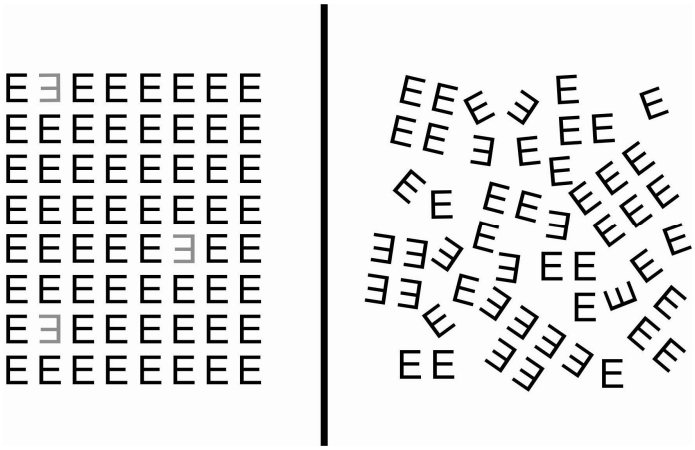


Figure 3. *A schematic representation of crystalline (left) and amorphous (right) states, with the symbol "E" representing a single molecule. Crystal defects are illustrated by using inverted gray symbols.*

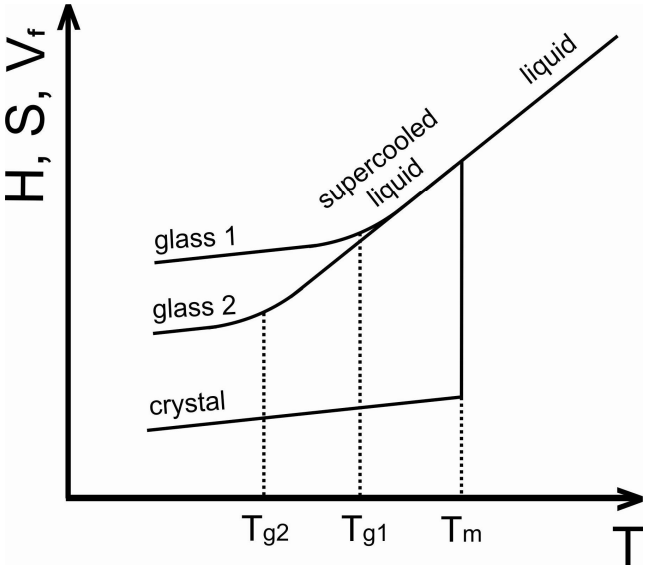


Figure 4. *Changes in H, S and V_f as functions of temperature for a material that can exist in either crystalline or amorphous form under T_g. Glass 1 is formed as a result of faster cooling than glass 2. Adapted from Ediger et al. (1996).*

As can be seen from Figure 4, cooling a liquid at different rates will produce glasses with differing T_gs and physical properties, such as H, S and V_f (Moynihan et al., 1974). This is because the cooling rate affects how much time individual molecules have to arrange, or "relax", towards the equilibrium conformation before they are apparently immobilized at T_g. As T_g is a kinetically controlled phenomenon which occurs over a temperature range without a fixed latent heat of transformation, it is not a 1st order phase transition (Kauzmann, 1948).

2.2.2 Molecular mobility of amorphous compounds

Even though the glassy state is physically more stable than the supercooled state, all glasses will relax towards the equilibrium enthalpy of the corresponding crystalline state when aged (annealed) under T_g (Ediger et al., 1996). Therefore, despite their different production methods, glass 1 in Figure 4 can theoretically be converted into glass 2 by annealing it for a certain amount of time. The relaxation rate is dependent on the structure of the glass, as well as the annealing temperature, and relaxation leads to a decrease in H, S and V_f as individual molecules and ordered molecule clusters reach a state of lower conformational energy. The structural relaxation time (τ), i.e. the time after which the intermolecular structure of a liquid/glass no longer bears resemblance to the structure at an earlier time point, is approximately 100 seconds at T_g and it increases rapidly when temperature is decreased. When relaxation proceeds, molecular mobility is simultaneously reduced due to a decrease in excess H, S and V_f in relation to the equilibrium (crystalline) state. Both the time and temperature dependence of relaxation rate may be modeled for example by using the Adam-Gibbs-Vogel equation (Adam and Gibbs, 1965), where the deviation of the glass structure from equilibrium is depicted by using the concept of fictive temperature (T_f). The equation may be presented as (Hodge, 1987):

$$\tau = \tau_0 \exp\left[\frac{DT_0}{T\left(1-\frac{T_0}{T_f}\right)}\right] \qquad (1)$$

In Equation (1), τ_0 is the pre-exponential factor (usually taken as 10^{-14} s), D is the so-called strength parameter and T_0 is the temperature where relaxation times approach infinity. D describes the deviation of a material's initial relaxation rate from Arrhenius temperature dependence, large values meaning "strong" materials that follow Arrhenius behavior, and small values "fragile", non-Arrhenius behavior following materials.

It should be noted, however, that there are several different ways to characterize molecular mobility. In addition to translational (global) mobility, molecules also possess rotational and vibrational (local) mobility and distinguishing between these may sometimes be difficult, especially when studying their combined effects on protein stability (see chapter 2.3.3). Still, all molecular mobility seems to affect the crystallization rate from amorphous state. Upon crystallization, intra- and intermolecular hydrogen bond networks of molecules form repeating motifs based on the most stable structure available and the overall energy of the system (Etter, 1990). Ordering of the molecules requires some degree of mobility, because in amorphous state the molecules are randomly oriented at “long” range. Maximum crystallization rate is normally observed between T_g and T_m, where rapid global mobility facilitates the reorientation of molecules (Bhugra and Pikal, 2008). On the other hand, it has been previously shown that crystallization may also be initiated at very low temperatures relative to T_g, in some cases even at T_g - 175°C (Okamoto and Oguni, 1996), where global molecular mobility should be extremely limited. This may mean that local mobility alone, such as the rotation of hydrogen bond forming moieties, may be sufficient to allow the alignment of stable motifs in the glassy state.

2.2.3 Water sorption and its effect on molecular mobility

In addition to the temperature effect described in the previous chapter, the molecular mobility of a glass can also be altered by mixing additional components into it. This is because mixing equal amounts of two miscible compounds will form a glass with a single intermediate T_g between the glass transition temperatures of the individual components (Hancock and Zografi, 1994). T_g lowering compounds are often referred to as plasticizers, and their addition increases molecular rotation and movement in the amorphous mixture. If a solute and a solvent, here a pure compound glass and a plasticizer, with different T_gs are mixed without a change in the overall volume of the system, T_g of the mixture will change according to the following equation:

$$T_g(mix) = \frac{w_1 T_{g1} + K w_2 T_{g2}}{w_1 + K w_2} \quad (2)$$

where T_{g1} and T_{g2} are the glass transition temperatures of the solute and solvent and w_1 and w_2 their weight fractions, respectively, and:

$$K = \frac{\rho_1 \Delta\alpha_2}{\rho_2 \Delta\alpha_1} \quad (3)$$

where ρ_1 and ρ_2 are the densities of the solute and solvent and $\Delta\alpha_1$ and $\Delta\alpha_2$ their increments of thermal expansion coefficient at glass transition, respectively. The material coefficient K gives the extent of T_g depression as a function of solvent content, with a higher value indicating a more steep change. Equation (2), known as the Gordon-Taylor equation (GT), was originally formulated to describe the glass transition temperature changes in polymer blends (Gordon and Taylor, 1952). However, it has since been successfully employed for modeling different kinds of amorphous mixtures as such, or with small modifications (Hancock and Zografi, 1994; Rodriguez Furlan et al., 2011). The GT model is highly useful in the prediction of T_g changes in two-component mixtures as a function mixing ratio, but its results are dependent on sample pretreatment and measurement parameters (Frank, 2007). Therefore, care must be employed when comparing values acquired from different literature sources.

One of the most common plasticizers in amorphous hydrophilic pharmaceuticals is water, which has a T_g of approximately -135°C (Haque and Roos, 2003). With such a low T_g, it is evident from Equation (2) that even a small increase in water content will significantly reduce the overall glass transition temperature of an amorphous mixture. When evaluating the effect of water as a plasticizer, it should be noted that the plasticizing effect of water may depend on how it interacts with the amorphous phase. Water in crystalline state, such as in ice or hydrate lattice, may be restricted from interacting freely with other components of the system. On the other hand, water that is mixed within the glass has the possibility of affecting its molecular mobility more. At a given temperature, the mobile water content of a system at equilibrium can be related to its water activity (a_w), which is commonly expressed as (Reid, 2007):

$$a_w = \left(\frac{p_w}{p_w^0}\right)_T \quad (4)$$

where p_w equals the equilibrium partial vapor pressure of water in a system and p_w^0 the equilibrium partial vapor pressure of pure liquid water, both in a given temperature (hence the subscript T). It should be stressed that a_w is not an absolute measure of water content, whether it is mixed within the amorphous phase or not, and it only describes the fugacity (escaping tendency) of water from the overall system. Changes in a_w can therefore be due to an alteration either in total water content (e.g. sorption/desorption), in water mobility (e.g. crystallization/melting), in sample hydrophilicity/-phobicity or in the porosity or dimensions of the overall sample.

Water sorption characteristics of a system under isothermal conditions can be modeled, for example, by using the Brunauer-Emmett-Teller (BET) equation, which predicts the sorption isotherms well for a_w between 0 - 0.55 (Brunauer et al., 1938). The BET model was originally created to model the adsorption of nonpolar gases on nonpolar surfaces, but it has since been also employed to model the water sorption of different amorphous saccharides (Zhang and Zografi, 2000). The model can be used to calculate, for example, the amount of water necessary to generate a monolayer of water molecules on the solid surface, which gives an estimate of available water binding sites on that surface. In its linearized form, the equation can be expressed as:

$$\frac{a_w}{(1-a_w)m} = \frac{1}{m_0} + \left(\frac{c-1}{m_0 C}\right)a_w \quad (5)$$

where m is the water content in grams per every 100 g of solids and m_0 is the amount of water in one monolayer in the same units. C is the heat constant of surface sorption, which has been hypothesized to be affected by the effect of water plasticization on the free energy of the amorphous system, as well as by the chemical affinity of water on solids (Zhang and Zografi, 2000). C-values between 2–50 are often observed in case of amorphous solids, whereas lower (< 2) are more common for crystalline materials (Labuza and Altunakar, 2007).

Water plasticization affects the molecular mobility of amorphous systems, which in turn is closely linked to protein stability in dry formulations. Therefore, studying how water uptake affects an amorphous formulation is of utmost importance when deciding what kind of processing and storage conditions are acceptable for that particular product.

2.3 Protein instability

Protein instability is a concept that can include both covalent bond breaking or formation in proteins that leads to the formation of new chemical entities (chemical instability), as well as non-covalent changes that result in alterations in secondary, tertiary and/or quaternary protein structures (physical instability) (Manning et al., 1989). This is, of course, not always a clear division, and distinguishing between the two may be difficult at times. Protein stability in different types of products is governed by a large number of factors, and introducing all of those currently known would be beyond the scope of this work. However, some of the most

important ones regarding aqueous solutions, as well as frozen and dried systems are discussed below.

2.3.1 Factors affecting protein stability in aqueous solutions

Proteins are often thermolabile, and their three dimensional structure tends to become unfolded (denaturated) above certain temperatures. In the simplest two-state model this can be described as (Pace, 1975):

$$N \rightleftarrows U \quad (6)$$

where N denotes the native and U the unfolded (or denaturated) state. The transition from native to unfolded occurs because an increase in temperature weakens the hydrophobic bonding, disrupts intramolecular ion pairs and strengthens the hydrophobic surface interactions, all of which affect the three dimensional structure of proteins (Jaenicke, 1991). When heated at a constant rate, the temperature where approximately 50% of proteins are unfolded is known as the "melting" or denaturation temperature (T_d), and the value is sometimes used to characterize the thermostability of a given protein (Wang, 1999). However, it is possible for a protein to have two or more denaturation temperatures depending on the experimental conditions and the analytical methods used. For example, several denaturation temperatures may be observed if the protein forms stable unfolded intermediates upon heating (Kosa et al., 1998) or if it contains several domains, each of which unfold individually (Remmele et al., 1998).

In addition, proteins can also be denaturated at low temperatures. This "cold denaturation" is thought to be caused by the hydration of non-polar moieties, which leads to somewhat similar secondary structure alterations as high temperature denaturation (Jaenicke, 1990). Despite the fact that proteins are subjected to low temperatures during freeze drying, cold denaturation does not appear to significantly contribute to protein instability during processing (Bhatnagar et al., 2007). In fact, some proteins may maintain their biological activity for extremely long periods of time when they are maintained in supercooled aqueous solutions, for example between -12 and -20°C (Hatley et al., 1987).

As protein structures are largely dependent on the balance between their hydrophobic and -philic moieties, it is no wonder that pH and ionic strength significantly affect their conformational stability (Goto and Fink, 1989). A change in pH of the surroundings can ionize a number of protein subgroups, and if these ionizing groups are located in the interior of folded proteins, they tend to destabilize protein conformations due to electrostatic repulsions (Yutani et al., 1987). The effect of ionic strength is dependent on pH (Goto and Fink, 1989), and the interplay between the two is often difficult to predict. Solution pH may also affect the rate at which chemical reactions leading to protein degradation progress, such as in the case of Maillard reaction in the presence of reducing compounds (see chapter 2.5.1) (Martins et al., 2001).

Mechanical stress may lead to structural perturbations in proteins. For example, antibodies appear especially susceptible to three dimensional structure alterations (Mahler et al., 2005), but the effects of different kinds of mechanical stresses on antibody stability, such as those caused by shaking or stirring, may vary (Kiese et al., 2008). The source of the mechanical stress factor has been attributed to the formation of an increased air/water interface area as bubbles are formed (Eppler et al., 2010). Shearing during processing might also denature proteins, but it has been calculated that the force exerted on proteins at air/water interfaces seems to be significantly higher than that of pure shearing (Bee et al.,

2009). Shaking-induced aggregation has been shown to be aggravated in the presence of silicon oil (Thirumangalathu et al., 2009), which is sometimes used in primary packaging as lubricant. The oil may also be transferred into the solution during processing, or during shipping when it comes into contact with the stopper.

If an aqueous solution contains other dissolved compounds besides the protein, these compounds may have an effect on protein stability. This is because in an aqueous protein solution that does not contain any additional solutes all the available surface sites of the protein are occupied by water molecules. However, when additional solutes (s) are present, some of the water molecules (H_2O) on protein surface sites (P) tend to be replaced by these molecules (Timasheff, 1998). Therefore:

$$P \times H_2O + s \overset{Kex}{\longleftrightarrow} P \times s + H_2O \qquad (7)$$

where K_{ex} represents the exchange coefficient:

$$K_{ex} = K_s/K_{H2O} \qquad (8)$$

where K_S and K_{H2O} are the solute and water binding constants, respectively. Protein conformation has an effect on K_S, and in some cases the solute interacts more strongly with the protein after protein unfolding has taken place (Timasheff, 1992). In such a case, the activation energy of protein denaturation is lowered by the addition of the solute, which shifts the overall balance of the reaction to the right in the two-state protein folding model depicted by Eq. (6). Examples of solutes that are preferentially bound to protein surface sites over water include urea and guanidine hydrochloride, but their effect on the denaturation activation energy seems to vary between different proteins (Pace, 1975). This is most likely due to differences in protein structures.

However, solutes may also stabilize proteins. If $K_{ex} < 1$, the solute in question is preferentially excluded from the surface sites, which leads to the preferential hydration (i.e. increased water interaction) of the overall protein surface. As a result, the chemical potential of the solution is increased, as the solute molecules are less likely to be located near the protein surface than farther away from it (Lee and Timasheff, 1981; Timasheff, 1992). Since protein unfolding is generally accompanied by an increase in surface area (Lee and Timasheff, 1981), denaturation is thermodynamically less favorable in the presence of preferentially excluded solutes, because it would lead to a further increase in the chemical potential of the solution. Examples of such solutes include disaccharides such as sucrose, as well as polyalcohols such as sorbitol (Timasheff, 1992). If a solution contains both preferentially bound and excluded compounds, their concentration differences and tendencies to interact with the protein define the overall effect on denaturation activation energy. It should be noted that preferential exclusion only affects solutions. If the solvent is removed, the concentration gradient disappears and preferential exclusion can no longer be used to describe protein stability (Allison et al., 1996; Carpenter and Crowe, 1989).

2.3.2 Factors affecting protein stability in freezing aqueous solutions

Cold denaturation (see previous chapter) must always be separated from freezing-induced protein stress. Firstly, freezing creates new liquid/solid interfaces into the solution, forming an electric potential difference gradient in the vicinity of the interface (Workman and Reynolds, 1950), that is potentially harmful for proteins. Another stress mechanism may

be direct protein adsorption to ice, which provides the thermodynamic driving force for unfolding by increasing the entropy of the system (Bhatnagar et al., 2007). This kind of adsorption can also take place at the water/container interface, but because of the small size of ice crystals, their surface area is considerably larger than that of the container in a freezing system. Still, even the ice surface area is finite, and it can be saturated with proteins or other compounds. The extent of surface-induced unfolding seems to be protein-dependent, but protein adsorption characteristics vary between different surfaces (Vermeer et al., 1998), such as glass or rubber. Freezing also reduces the amount of liquid water in the system, which in turn increases the concentration of all solutes. This can lead to an increase or decrease in protein stability, depending on the solute in question. In some cases, higher protein concentration may decrease the extent of unfolding during freezing (Allison et al., 1996; Liu et al., 2005). One explanation for this is that if the protein concentration of the aqueous solution is relatively high before freeze concentration, freezing may increase it to a point where the proteins inhibit each other's molecular movement through steric hindrance. Another explanation is that proteins are unfolded at the ice/water interface and a high enough protein concentration can saturate that interface, which protects the protein molecules that are not in contact with the ice/water layer.

If the freezing solution contains buffers that have a tendency to crystallize, freeze-concentration may lead to pH shifts that can be harmful for proteins (see chapter 2.3.1). Similarly, compounds that increase protein stability in aqueous solutions through preferential exclusion may crystallize during freeze-concentration, revoking their protective effect and forming solid/liquid interfaces which may further destabilize proteins (Piedmonte et al., 2007). Finally, the freeze-concentration of the protein itself may result in a high concentration phase, where the protein solubility limit is exceeded, or cause the attractive protein surface forces to exceed the repulsive ones, leading to higher rate of chemical reactions and/or aggregation (Shire et al., 2004).

2.3.3 Factors affecting protein stability in amorphous state

When aqueous protein formulations are dried, the water surrounding proteins is removed either partly or completely. Water acts as a stabilizing component for proteins, since it keeps individual polypeptide chains apart and helps to conserve their three dimensional structure. The amount of water required for full hydration has been approximated to be less than 0.25 g per one gram of protein (Jaenicke, 1991). Removing a part of the water surrounding the proteins may cause some ionized moieties to lose their charge, which decreases the activation energy of hydrophobic interactions between neighboring proteins and can lead to conformational alterations. Such harmful dehydration effects may be countered, or made reversible, if the initial formulation contains compounds that are able to form hydrogen bonds with protein surfaces (Carpenter and Crowe, 1989). These compounds may partly replace water around the protein, and mimic the effects of the hydration shell that is necessary for upholding the three dimensional structure close to native. Such action seems to result in an improved retention of the biological activity of proteins (Prestrelski et al., 1993a), and molecules that contain many hydrogen bond forming moieties, such as carbohydrates, tend to be suitable for this purpose. On the other hand, larger polymeric molecules might not be able to form a sufficient amount of hydrogen bonds with the protein due to steric hindrance, which may make some of them less effective hydrogen bond replacing compounds (Tanaka et al., 1991). It also seems that even the most stabilizing compounds cannot completely replace all water of the hydration shell. Therefore, protein structure may be altered if the residual water content of the dried product decreases too low.

For example, a dried antibody product with an intermediate water content (≈ 3%-w/w) has been shown be more stable during storage than those containing less or more water than this (Chang et al., 2005).

If a solution contains solutes (besides the protein-structured API) that do not crystallize from supercooled solutions at subzero temperatures under experimental time scale, these may form a glassy matrix during cooling and freeze-concentration that envelops proteins. Such compounds can therefore be used to encase proteins within a viscous matrix, which limits the molecular mobility of the protein, and may slow down the interconversion between conformational states (Hagen et al., 1995). The role of the viscous matrix in protein stabilization, however, is still not completely understood. The molecular mobility of an amorphous matrix at temperatures under T_g may be divided into translational (global) and vibrational (local) mobility, as explained in chapter 2.2.2, and their individual and combined effects on protein stability are yet to be elucidated. However, it has been hypothesized that the effect of global molecular mobility on the storage stability of dried amorphous protein products may be sometimes limited (Yoshioka and Aso, 2005), whereas local mobility may play a more significant role (Chang et al., 2005; Yoshioka et al., 2007). The plasticizing effect of residual water content (described above) must also be taken into account when determining the molecular mobility of an amorphous phase, as explained in chapter 2.2.3.

Naturally, the crystallization of a protective glass forming compound will exclude proteins from its vicinity and decrease its protective efficacy, as proteins cannot be included within the ordered crystal lattice of the protective compound (Izutsu et al., 1993). Similarly, crystallization reduces the amount of heteromolecular hydrogen bonds that the protective compound may form, which makes it less effective in replacing water as a hydrogen bond former with the hydrophilic protein surface (Carpenter and Crowe, 1989).

2.3.4 Protein aggregation and fragmentation

Chemical and physical protein instability may lead to a number of different outcomes, but one of the most distinguishable ones is certainly aggregation. It is a relatively common response to most stress factors, since any kind of transition in protein structure that leads to a change in the attractive and repulsive forces between protein molecules, including the formation of intermolecular covalent bonds, may possibly lead to aggregation (Wang, 1999). However, due to the complexity of protein structures, two aggregates are never completely alike. Protein aggregate classification systems based on their size, reversibility of formation, conformation, possible chemical modification and morphology have been suggested by several authors, such as Mahler et al. (2009) and Narhi et al. (2012), as shown in Table 1. Classifying the aggregate profile found from pharmaceutical products is important, because differences in their size, chemical composition and surface properties may cause a change in their biological response (see chapter 2.3.5). There are a number of different aggregation pathways that proteins may undertake, some of which are shown in Figure 5. It should be noted that this clearly differs from the simple two-state model described in Eq. (6), where no intermediate formation is considered due to sake of simplicity. Proteins can aggregate directly from the native state, as shown by pathway 2, either by forming potentially reversible electrostatic and/or hydrophobic interactions (2a) or through practically irreversible covalent crosslinking (2b) in Figure 5. Partial structural deformation (i.e. intermediate formation) prior to aggregation also appears to be common, whereas aggregation through completely denatured state may be more rare, which is represented by the gray arrows. Disulfide bridge formation/exchange plays an important role in covalent crosslinking (Carba et al., 2008), potentially leading to native or denatured protein aggregation either directly through

pathway 2b or indirectly through pathway 3. However, other chemical reactions such as the Maillard reaction can also have a similar result as disulfide bridging (Wang et al., 2010). Even though protein aggregates may initially be soluble, they may eventually precipitate as amorphous or ordered structures, once a certain size or solubility limit is exceeded.

Table 1. *Properties that can be used to describe and divide aggregates into different subclasses. Table is based on the classification systems of Mahler et al. (2009) and Narhi et al. (2012).*

Property	Subclasses
size	small oligomers, large oligomers (≥10-ers), soluble particles with Ø = 20 nm – 1 µm, insoluble particles with Ø = 1 - 25µm, large visible particles
reversibility	Reversible, irreversible, dissociable (under given conditions)
conformation	Predominantly native, predominantly non-native
chemical modification	Cross-linked, intramolecular modification, oxidation etc.
morphology	Number of monomers, aspect ratio, surface roughness etc.

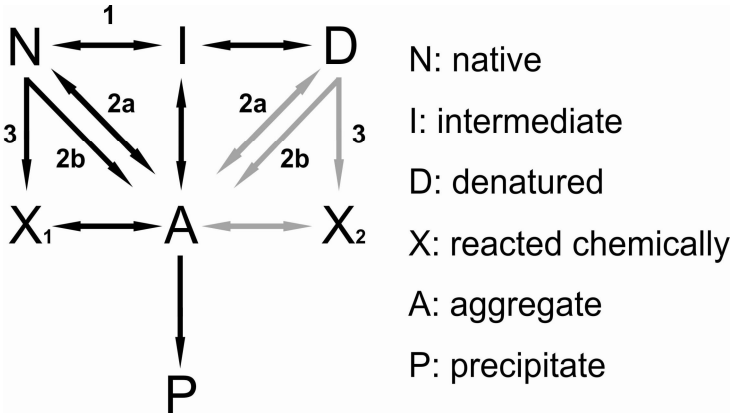


Figure 5. *A general scheme representing possible protein aggregation pathways. Pathway 1 represents aggregation through intermediate unfolding, pathway 2 through protein self-association and pathway 3 through chemical linkage formation. Subscripts a and b stand for reversible and irreversible aggregation, respectively. Black arrows represent primary (more common) and gray arrows secondary (less common) pathways. Adapted from Wang et al. (2010).*

In addition to aggregation, proteins may also undergo fragmentation to form units that are smaller than one monomer in size. This is relative common in antibodies such as immunoglobulin G (IgG), where the protein monomer is actually a homodimer of two heavy chain/light chain pairs, and these can relatively easily be separated into smaller subunit, for example by extreme pH conditions (Usami et al., 1996), high temperatures (Alexander and Hughes, 1995) or freeze-thawing (Paborji et al., 1994). This will reduce the active monomer content, possibly decreasing the efficacy of the product and causing structural alterations which may lead to immune responses (see next chapter).

2.3.5 Product-related immunogenicity

Non-native proteins may cause an immune response when entering the blood stream. When this happens, either with or without being activated by helper T-lymphocytes, B-lymphocytes begin producing antibodies that bind specifically to a certain epitope of the non-native protein (Rosenberg, 2006). It would appear that the epitopes that activate B-lymphocytes may be expressed not only in the linear amino acid sequence, but in the three-dimensional protein conformation as well (Ito et al., 2003). This would suggest that alterations even in the conformation of fully human proteins might turn them immunogenic. Also, the formation of protein aggregates may create three-dimensional structures that are not expressed in native-form proteins, which has led to the hypothesis that such aggregates might be immunogenic.

There are most likely several factors that determine the prevalence and nature of immune response in patients caused by a biopharmaceutical drug product. Based on non-clinical data, it has been suggested that products with higher aggregate content might trigger a stronger immune response than those with lower content (Gamble, 1966). On the other hand, in a more recent non-clinical study it has been suggested that the structure and formation method of aggregates affects their immunogenicity more than their quantity or size (Filipe et al., 2012). It has been hypothesized that differences in aggregation methods might affect their surface structure as a result of monomer unfolding, reordering and chemical modification, and certain structural features such as repetitive epitope ordering might induce a stronger immune responses than others (Filipe et al., 2010b). In extreme cases, the immune response caused by a drug product can lead to life-threatening conditions such as anaphylactic shock, but immunogenicity might also lead to reduced therapeutic efficacy in long term administration (Rosenberg, 2006). This is because sometimes the immune system creates anti-drug antibodies only against the unfolded proteins, but on some occasions it may adapt to activate against the native form as well (Vazquez-Rey and Lang, 2011). The resulting reaction may therefore inactivate a portion of the dose and reduce the efficacy of the drug. Whether this is commonly clinically relevant, however, is unclear.

It should be noted that direct clinical evidence identifying aggregates as a major contributing factor in the immunogenicity of biopharmaceutical products is still missing (Singh et al., 2010). Furthermore, it is often difficult to find reliable causes for immunogenicity differences between protein drug products, as there are several factors contributing to this. For example, there may be significant differences in the immunogenicity of drug products containing structurally similar proteins. In one such case, the percentage of patients developing neutralizing antibodies against the protein-structured API was 13% for an interferon (INF) β-1a product (Avonex®, Biogen IdeC Inc., Weston, MA, USA) and 43% for an INF β-1b product (Betaferon®, Bayer HealthCare Pharmaceuticals, Berlin, Germany) (Sominanda et al., 2007). Comparing the two products is made difficult by the fact that besides containing APIs with different protein structures, Avonex® is an aqueous solution that is administered intramuscularly, whereas Betaferon® is freeze-dried and administered subcutaneously. It is also noteworthy that in the study of Sominanda et al. (2007) Avonex® and Betaferon® were administered to different patients. This is relevant, because the immune systems of different patients may react differently to a given product (Jahn and Schneider, 2009), meaning that there may be differences in the apparent immunogenicity of a drug product depending on the patient group to which it is being administered.

Based on the possible effect of protein aggregation on product immunogenicity, the measurement of product aggregation profiles is currently under intense discussion in the pharmaceutical literature. From the regulatory point of view, the guidelines that deal with

aggregate content quantification, such as the Ph.Eur chapter 2.9.19 and the USP chapter <788>, only give maximum allowed particle content limits for particles larger than 10 µm in size. Consequently, they have been criticized for not being fully descriptive of the quality of biotechnological products on the basis that smaller aggregates than this may still be immunogenic (Carpenter et al., 2009). Furthermore, other aggregate properties besides size are not controlled. However, if the effect of protein aggregation on immune response is to be estimated, accurate qualitative and quantitative aggregate analysis methods are required. This represents a serious problem in the current analytical technologies, because especially in the case of submicron aggregates, factors such as sample pre-treatment and measurement method heavily affect the results (Singh et al., 2010). The lack of universally accepted analysis methods that could differentiate between protein aggregates, silicon oil droplets and air bubbles may therefore make it difficult to set regulatory limits for submicron particles present in drug products. Therefore, International Conference on Harmonization (ICH) guideline Q6B on the test procedures and acceptance criteria for biotechnological products states only that these products should be fully characterized to ensure their safety and efficacy (ICH, 1999). Resolving a suitable methodology for this must be done by the manufacturer on a case-by-case basis (Jahn and Schneider, 2009).

2.4 Freeze drying

Freeze drying, or lyophilization, is a common method for drying heat sensitive materials, such as biological samples and proteins. It consists of dissolving the compound(s) to be freeze dried in a solvent, freezing the solution and then removing the crystalline solvent through sublimation and evaporating the non-crystalline solvent. From here on only the freeze drying of aqueous solutions is considered, even though it is possible to use other solvents that are liquids in room temperature for compounds that do not dissolve in water. Freeze drying is commonly divided into three stages, freezing, primary drying and secondary drying, and the shift from one stage to the next can usually be identified as the point when the main type of phase transition occurring in water changes (e.g. from crystallization to sublimation). An illustration of an imaginary conventional freeze drying cycle is shown in Figure 6. It should be noted, however, that temperatures and drying times tend to vary significantly between cycles.

Although there are several models available on the market, industrial scale freeze dryers always tend to contain temperature-controllable shelves where the product vials are kept, a condenser and a vacuum pump. The shelves can be used to cool the solutions to be freeze dried, resulting in ice crystal formation, and to affect product temperature and water removal rate during all process stages. The condenser is needed to remove sublimed water from the air by deposition, and the vacuum pump is necessary to control chamber pressure during primary and secondary drying. The three stages that make up the freeze drying process, freezing, primary drying and secondary drying, are explained in detail below.

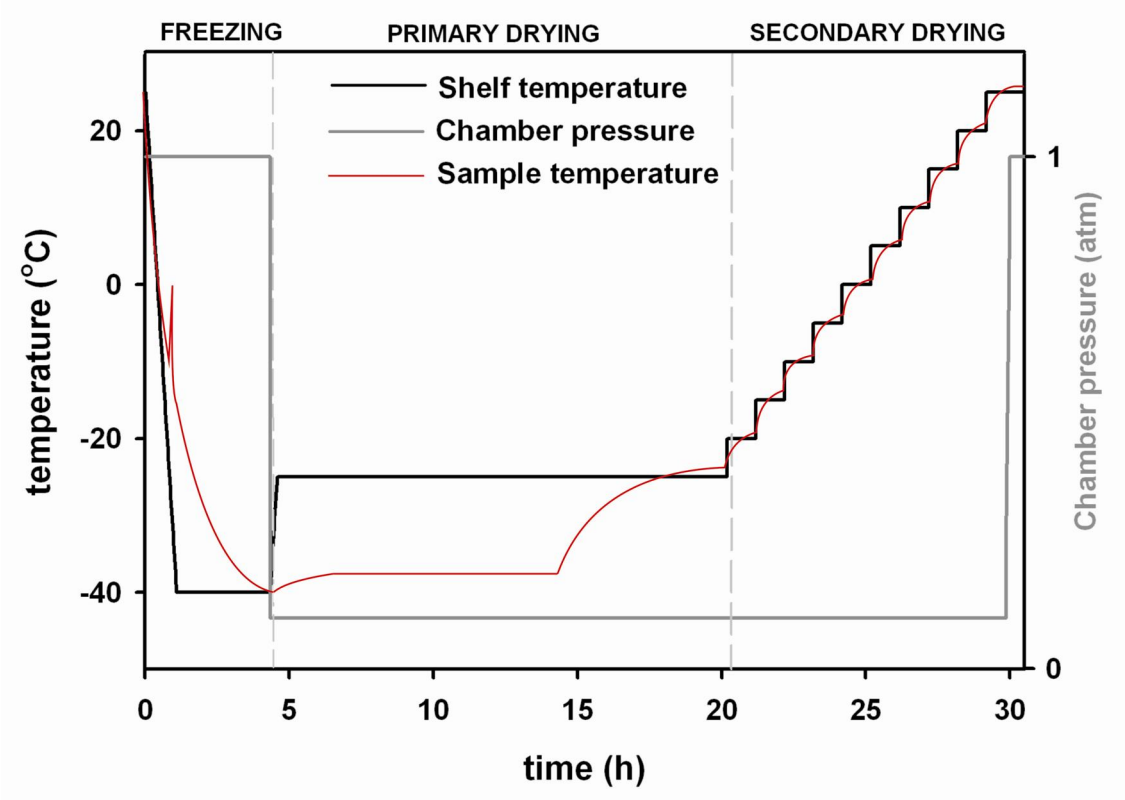


Figure 6. *A depiction of a fictional freeze drying cycle, where the effects of shelf temperature and chamber pressure on sample temperature during drying are shown during different stages of drying.*

2.4.1 Freezing

To begin a freeze drying process, the temperature of an aqueous solution containing the API and excipients (solutes) must be lowered in order to turn the majority of liquid water (solvent) to solid ice crystals. The part of the freeze drying process leading to the formation of this solid intermediary product is generally referred to as the freezing phase, and the phase transitions occurring during it are depicted in Figure 7. Upon cooling, the aqueous solution does not normally freeze at the equilibrium freezing temperature of the solution (denoted by [I] in Fig. 7), as crystallization is a stochastic phenomenon (Franks, 2007). The extent of how much the temperature of the solution decreases below its equilibrium freezing temperature before the appearance of ice crystals is known as the degree of supercooling. When ice nucleation does occur, the extent of supercooling determines the growth rate of ice crystals. This is because the driving force for crystallization is higher when the solution is more supercooled, and a higher degree of supercooling will therefore result in faster nucleation rate and smaller average crystal size (Kasper and Friess, 2011). As higher extents of supercooling are usually achieved by using slower cooling rates, very fast cooling may conversely lead to slow freezing rates.

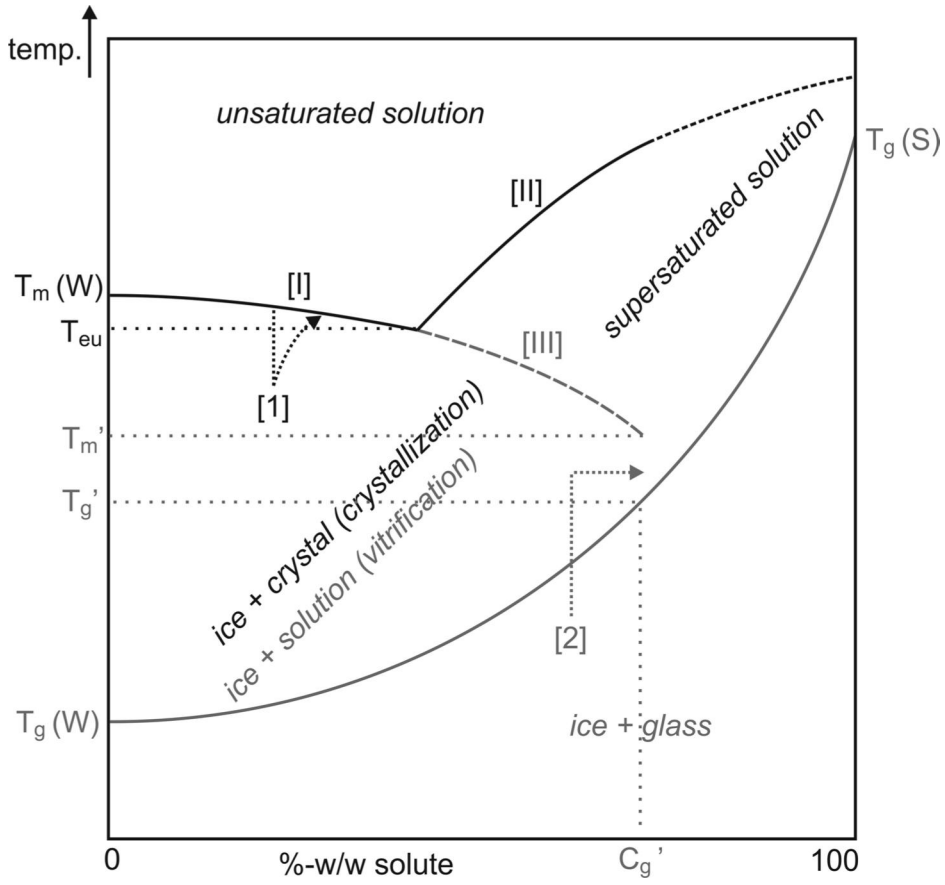


Figure 7. *State diagram of a system containing water (W) and solute (S).The explanations of markings [I] through [III] and [1] through [2] are given in text. The major physical states present and phase transitions occurring under different conditions are marked with either black or gray, depending on whether the predominant behavior of the solute under experimental time scale in solutions at that temperature is crystallization or vitrification, respectively. Adapted with changes from Liu et al. (2005).*

Ice crystal formation increases the temperature of the dispersion due to the latent heat release of crystallization, and the removal of water from the solution increases the concentration of solutes (marked with [1] in Fig. 7). When the dispersion freezes further, the concentration of solutes follows the equilibrium freezing curve (marked with [I]) until the equilibrium solubility curve (marked with [II]) is met. At this temperature (known as the eutectic temperature, T_{eu}) the concentration of readily crystallizing solutes becomes high enough to allow crystallization, but like in the case of ice formation, this phase transition tends to occur at a temperature below T_{eu}. The readiness of supersaturated solutes to crystallize is dependent on the complexity of their crystal structure, and in the case of compounds such as disaccharides this structure is so complex that they do not normally crystallize from supersaturated solutions under experimental time scale (Kasper and Friess, 2011). In such cases cooling the solution will cause further freeze concentration along the equilibrium concentration curve (marked with [III] in Fig. 7). When cooled to a sufficiently low temperature, the viscosity of the solution will eventually exceed the point where the translational mobility of solute molecules becomes negligible. This point is known as the glass transition temperature of the maximally freeze-concentrated amorphous phase (T_g'), and due to the drastic reduction in molecular mobility, ice crystal growth no longer takes place at meaningful rates at temperatures below it. As freezing may turn more than 99% of

the total water present in the original solution into ice, the solute concentration of the maximally freeze-concentrated amorphous phase (C_g') tends to be independent on the initial solute concentration (Franks, 1998), except in very dilute solutions.

After the solution has been frozen below T_g', no significant changes in ice crystal size or solute concentration are expected to occur under experimental time scale. However, if the solution has been cooled very quickly (e.g. by using liquid nitrogen quenching), solute concentration may not have increased along the equilibrium concentration curve (marked with [III] in Fig. 7), as freeze-concentration takes time. Therefore, the solute concentration will be below C_g'. This means that the T_g of the solute phase is now lower than T_g' due to its higher water content, as dictated by the Gordon-Taylor equation (Eq. (2) in chapter 2.2.3), and this may pose problems during primary drying (see next chapter). In such cases the solution temperature may be increased above its glass transition temperature to allow further freeze-concentration to take place (marked with [2] in Fig. 7). However, care must be taken not to exceed the onset melting temperature of ice (T_m'), above which the ice starts turn into liquid water, causing a decrease in solute concentration and the amorphous phase.

This heat treatment method, known as annealing, can also be used to affect the ice crystal size after freezing has already taken place. Holding the temperature between T_g' and T_m' increases molecular mobility, allowing the interchange of molecules between solid ice and liquid water phases to take place. Ice crystals with relatively small radius of curvature tend to shrink, eventually disappearing completely, while large crystals grow (Searles et al., 2001). As a result, the average ice crystal size increases and size distribution decreases. This phenomenon is known as Ostwald ripening, and as its result, annealing can be used to increase and homogenize the average ice crystal size of frozen samples. It may also decrease primary drying time, as sublimation of the large ice crystals forms large pores which offer less resistance to water removal than small pores. Furthermore, narrowing the ice crystal size distribution may produce less variation between vials when a large number of samples are freeze dried simultaneously. Finally, annealing may be used to complete the crystallization of unstable excipients such as mannitol, when they are meant to crystallize and serve as bulk formers in the lyophilizate (i.e. the end product of freeze drying process).

Varying the cooling, and subsequently the freezing rate may have an effect on protein stability. Freezing solutions quickly by cryogen quenching (e.g. using liquid nitrogen) tends to lead to the formation of a large ice surface area due to the formation of many small ice crystals, and this surface area can act as an adsorption site for proteins such as bovine IgG (Sarciaux et al., 1999), as explained in chapter 2.3.2. The relationship between freezing rate and the extent of protein stress may be depend on the protein that is being frozen, however, as freezing lactate dehydrogenase by liquid nitrogen quenching has been shown to cause less protein activity loss than freezing it slowly on the shelves of a freeze drier (Nema and Avis, 1993). A reason for this may be that since fast freezing decreases the extent of freeze concentration of the solute phase, it may be less harmful to proteins which are susceptible to structural alterations in highly concentrated solutions.

2.4.2 Primary drying

After freezing the solution, ice is removed from the product by sublimation, i.e. by transforming solid water to vapor, which is carried out in a stage commonly referred to as primary drying. Since the crystalline phase tends to contain most of the total water in the sample (the rest being in unfrozen state in the freeze-concentrated amorphous phase), primary drying is usually the most time-consuming part of the freeze drying process, and different approaches are frequently considered to reduce its length as much as possible

(Chang and Fischer, 1995; Chatterjee et al., 2005; De Beer et al., 2007). Sublimation can be initiated by decreasing drying chamber pressure and increasing shelf temperature in such a way that the triple point of water is not exceeded. Sublimation of ice takes large amounts of energy (≈ 2.8 kJ/g), meaning that the subliming ice front cools down significantly if additional heat is not supplied. Heat can be transferred from the surroundings through three mechanisms, radiation, conduction and convection, of which convection has the greatest effect on product temperature during primary drying (Franks, 2007). Still, even though chamber pressure affects the rate of sublimation, it is not the driving force. This force is provided by the vapor pressure difference between the sublimating water front, and the condenser surface where water deposits. Therefore, condenser temperature should normally be a low as possible, since increasing the temperature difference between sublimation and deposition fronts will improve the rate of sublimation.

Carrying out primary drying so that the amorphous phase remains in highly viscous state will result in the formation of a porous lyophilizate structure, as shown in Figure 8, where the pores are formed after ice crystals sublime. Normally, product temperature is kept close to or at T_g' during primary drying (Tang and Pikal, 2004). This is because a higher product temperature allows the material to be dried faster, reducing production costs. On the other hand, exceeding T_g' may bring the temperature close to the collapse temperature (T_c). This temperature represents the point where the viscosity of the amorphous phase becomes sufficiently low to allow it to flow significantly under experimental time scale. T_c may in some cases be observed as close as 2°C above T_g', and exceeding it will cause the pores to start closing and the overall dimensions of the lyophilizate to decrease (so-called macrocollapse, or simply collapse). Macrocollapse may decrease drying rate during primary and secondary drying, since the porous structure can improve drying (especially when using large vial fill volumes), and collapse will also make the product visually unappealing. Exceeding T_g' during primary drying has also been suggested to have a negative effect on the stability of protein-structured pharmaceuticals, but this theory has been called into question in several recent studies (see below).

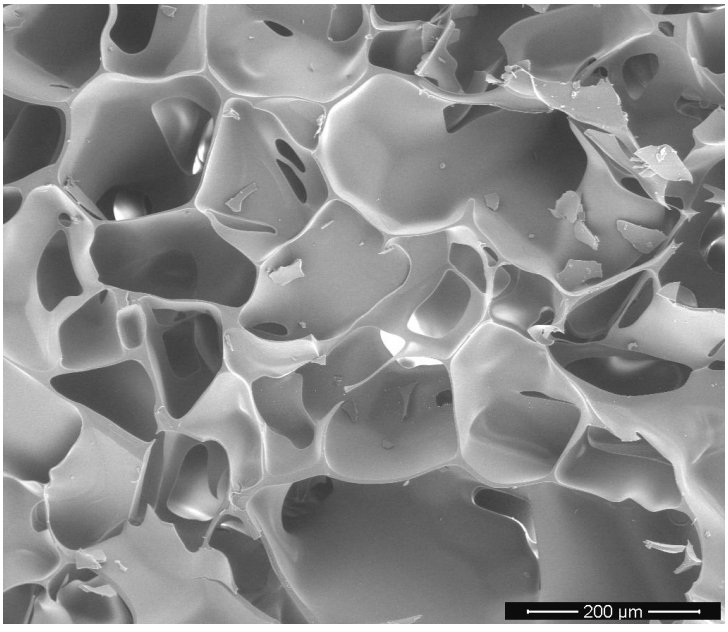


Figure 8. *A scanning electron microscope image of freeze-dried melibiose (magnified x350), which shows the porous structure that has been formed after ice crystals have sublimed.*

The interplay between shelf temperature and chamber pressure is illustrated in Figure 9, which depicts an imaginary system undergoing primary drying. If the system is being dried on shelves that are being kept at temperature X, under the chamber pressure of 45 A.U. (arbitrary units), the heat transfer balance keeps product temperature close to T_g' (point A). Reducing the chamber pressure to 22.5 A.U. will cool the sample by over 5 K, but it will also reduce sublimation rate due to lower product temperature, as heat convection slows down (point B). Increasing the chamber pressure to 60 A.U. will increase sample temperature almost 2.5 K above T_g' and improve sublimation rate, as heat convection becomes more effective (point C). However, this increase in temperature may bring product temperature close to T_c and result in product collapse. In a conventional freeze drying cycle shelf temperature and chamber pressure are normally optimized to maximize sublimation rate, while keeping product temperature a few degrees below T_c (point D).

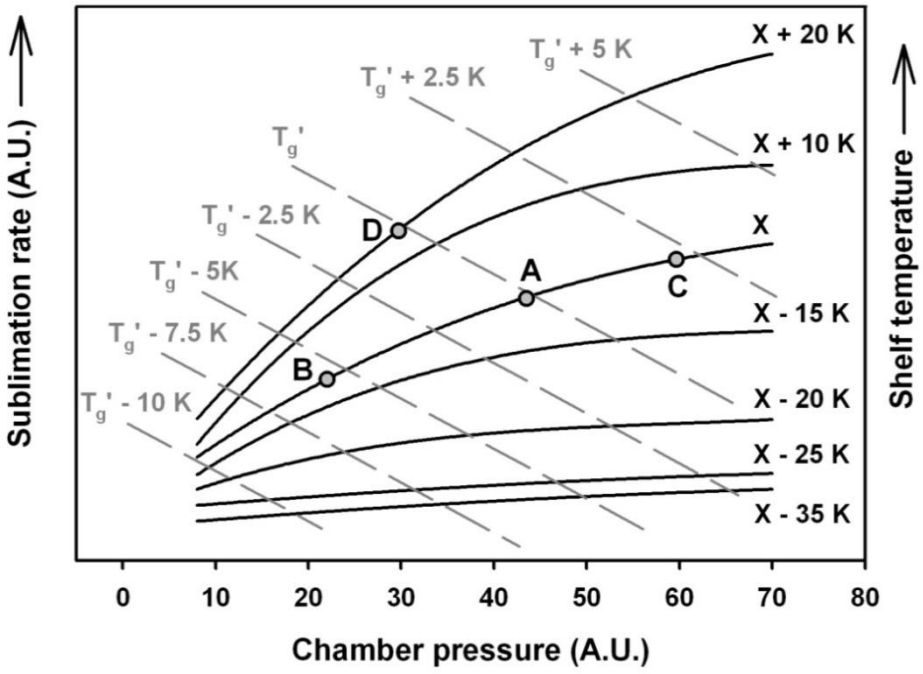


Figure 9. *The effect of shelf temperature and chamber pressure on product temperature (marked with gray text and dashed lines) and sublimation rate during primary drying. The explanations for markings A through D are given in text. Adapted with changes from Chang and Fisher (1995).*

According to the vitrification hypothesis, the high viscosity of the amorphous phase slows down the rate of chemical reactions and conformational changes of proteins at temperatures under T_g, thus minimizing changes to the native structure during processing, as explained in chapter 2.3.3 (Hagen et al., 1995). For this reason it has been assumed that exceeding T_g' during primary drying might increase the rate of chemical deterioration in proteins (Franks, 1998). However, several more recent studies have found no clear difference in the extent of protein degradation between samples that have been lyophilized below or above their T_g' during primary drying (Chatterjee et al., 2005; Sarciaux et al., 1999; Schersch et al., 2010). Furthermore, it seems that in some cases the storage stability of protein-structured pharmaceuticals is even better in collapsed lyophilizates than in non-collapsed ones (Schersch et al., 2012). This effect has been attributed to the reduction of the molecular

mobility of the glassy state, that occurs as a result of holding the amorphous mixture above its T_g during drying, as explained in chapter 2.2.2. Exceeding the glass transition temperature at some point during freeze drying may still have a deleterious effect on protein stability, however, if the formulation contains only very unstable lyoprotective excipients such as mannitol, that have a tendency to crystallize easily when held above their T_g (Izutsu et al., 1994).

2.4.3 Secondary drying

After primary drying the lyophilizate consists of solutes that had originally been dissolved in water, along with residual water that is either present in solid amorphous phase as "plasticizer" or as hydrate in crystalline phase. The main purpose of the third processing step, referred to as secondary drying, is therefore to define the amount of the residual water that remains in the final product. Water content reduction during secondary drying seems mainly to be limited by the rate of water evaporation from lyophilizate surface, even though the effect of water diffusion from lyophilizate core to surface may also be significant (Pikal et al., 1990). Due to this, product surface area has a greater effect on secondary drying rate than lyophilizate thickness. However, it is difficult to decrease overall freeze drying times by increasing the product surface area, because as stated in chapter 2.4.1, producing ice crystals with smaller average size might lead to decreased sublimation rate during primary drying (Searles et al., 2001). Therefore the net effect of decreasing the pore size on the overall drying time might even be negative. Furthermore, increasing the diffusion coefficient of water in a given formulation without compromising the structural integrity of the lyophilizate (i.e. causing collapse) would be challenging. Contrary to common beliefs, chamber pressure does not appear to have a significant effect on the drying rate during secondary drying in porous lyophilizates (Pikal et al., 1990). This is because once evaporated, water is quickly transported through the pores and towards the condenser.

Drying temperature should be increased in order to facilitate water evaporation, as the rate of water evaporation decreases as a function of time under isothermic conditions (Pikal et al., 1990). However, as the amorphous phase still contains a significant amount of water after primary drying, it is possible to induce partial or complete collapse in the lyophilizate by increasing the shelf temperature too rapidly (Franks, 2007; Shalaev and Franks, 1995). In order to optimize the effectiveness of a conventional secondary drying step, lyophilizate temperature may be increased as water is removed to keep it between T_g and T_c, as shown in Figure 10. When drying heat-labile proteins, the use of higher secondary drying temperatures or extending the time the formulation spends in high temperatures may result in increased protein aggregation (Franks, 2007). On the other hand, using lower temperatures extends the time taken to reach adequate residual water content, which may also an increasing effect on API degradation (Pikal et al., 1990). Since overdrying can also have a harmful effect on the stability of certain proteins (Chang et al., 2005), maximal secondary drying conditions are defined by the formulation in question.

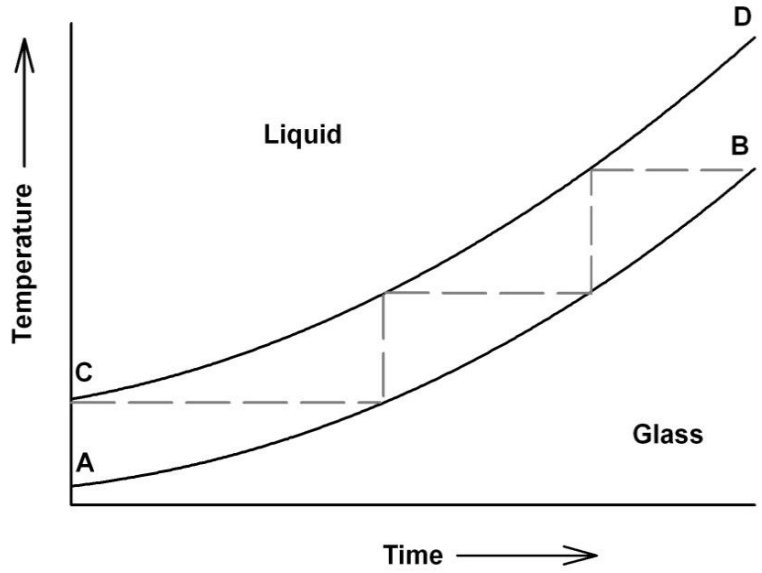


Figure 10. *The change in lyophilizate T_g (line AB) and T_c (line CD) as a function of secondary drying time (or decreasing residual water content). A near-optimal shelf heating rate of a conventional freeze drying cycle is depicted by the gray dashed line. Adapted with changes from Franks (2007).*

2.4.4 Significance of freeze drying as a protein stabilization method

As should be clear after this chapter, freeze drying may subject a protein to a number of different stress factors. In fact, many pharmaceutically relevant proteins can be made sufficiently stable in properly formulated aqueous solutions for them to reach an acceptable shelf life of 2-3 years at 2-8°C, whereas freezing and dehydration may be harmful for them. This, coupled with the fact that freeze drying is a cost-intensive batch-processing method, means that it is commonly not the first choice when selecting a stabilization method.

However, there are reasons why approximately 1/3 of all mAb drug products that have received marketing authorization are produced by freeze drying. Some proteins simply are not stable for long periods of time as aqueous solutions, meaning that drying is the most feasible option to produce a stable product that can be marketed. Also, one drawback of aqueous formulations is their susceptibility to shaking-induced degradation, as explained in chapter 2.3.1, but this should not occur in freeze-dried products before rehydration. It is also possible to develop freeze-dried protein pharmaceuticals that are stable in room temperature, such as in the case of the human plasma IgG extract Gammagard® (Baxter International Inc., Deerfield, IL, USA) and the INF-β1a product Avonex® (Biogen IdeC Inc., Weston, MA, USA). On the other hand, aqueous protein products must typically be kept refrigerated, making their transportation and storage more difficult. Compared to other drying methods of protein-structured pharmaceuticals, such as spray drying, freeze drying possesses the advantages of being commonly accepted by regulatory agencies, allowing relatively easy incorporation into aseptic manufacturing lines and not subjecting the product to high temperatures. Furthermore, freeze drying does not normally result in material loss during processing, which is not always the case in spray drying. While freeze drying affects the stability of different proteins in a different way (Prestrelski et al., 1993a), and may therefore not be suitable for all protein-structured APIs, it remains a highly important processing tool in the pharmaceutical industry.

2.5 Excipients used in freeze-dried pharmaceutical products

Protein-protecting excipients in lyophilized formulations are sometimes classified as cryo- and/or lyoprotective compounds, depending on whether they protect the API during freezing and/or drying, respectively. Theoretically, a cryoprotective excipient could be preferentially excluded from the vicinity of the protein, as explained in chapter 2.3.1, and/or it could have an amphiphilic molecular structure which allows it to concentrate on ice/water interfaces, as explained in chapter 2.3.2. On the other hand, a lyoprotective excipient could form a viscous glass during freeze-concentration and thus inhibit the molecular mobility of the protein, and/or partially replace water as hydrogen bond former on the hydrophilic surface of the protein, as explained in chapter 2.3.3. Naturally, a single compound may possess both cryo- and lyoprotective properties.

The mechanical strength of crystalline compounds is often higher than that of amorphous ones due to more organized bond structure. For this reason, easily crystallizing excipients, sometimes referred to as bulking agents, can be used to bring mechanical stability to otherwise easily crumbling lyophilizates (Shalaev and Franks, 1996). Furthermore, they can be used in combination with non-crystallizing protein-protecting excipients to uphold the porous lyophilizate microstructure even when T_c of the amorphous phase is exceeded during drying (resulting in so-called microcollapse). Employing such drying cycles might have the benefit of reducing the molecular mobility of the amorphous phase due to the annealing effect, but without significantly lowering drying rates because of decreased lyophilizate porosity and surface area.

Proteins often have an optimal pH range where they are most stable, and it is therefore commonplace to add buffering compounds to the solutions to be freeze dried (Wang, 2000). As most biopharmaceuticals are administered via injection or infusion (usually IV, SC or IM route), it is also necessary to make the formulation as close to isotonic as possible. Both pH and tonicity can be adjusted with excipients, but the effects of stability and freeze-concentration on solution properties must be taken into account. An extensive knowledge of the physicochemical properties of the API and excipients is therefore an integral part of the development process. Some of the most commonly used excipients are introduced below.

2.5.1 Low molecular weight saccharides and polyalcohols

Perhaps the most effective cryo- and lyoprotective excipients used in freeze-dried protein products are disaccharides. Commonly abundantly present in organisms that often experience dehydrating conditions (Crowe et al., 1996), disaccharides such as sucrose and trehalose have been studied thoroughly and they are well tolerated also in high doses. Furthermore, they can act as both cryo- and lyoprotective excipients, as they are preferentially excluded in aqueous solutions (Timasheff, 1998) and form viscous glasses (Hagen et al., 1995) with a high hydrogen bonding potential with the protein in solid state (Carpenter and Crowe, 1989). The disaccharides used in freeze-dried mAb products are currently limited to sucrose and trehalose (Wang et al., 2007), but maltose is used in the aqueous tositumomab / I131 tositumomab drug product Bexxar® (GlaxoSmithKline plc, Middlesex, UK). The glass-forming properties of disaccharides distinguish them from monosaccharides, whose freeze-concentrated aqueous solutions generally exhibit too low T_g' for them to be useful as freeze drying excipients in conventional formulations (Roos, 1997), where lyophilizate collapse is an undesired phenomenon. Similarly, even though polyalcohols are effective hydrogen bond formers and may thus protect proteins from dehydration-

induced stress, their high crystallization tendency reduces their suitability as the only lyoprotecting excipient in a formulation. However, their tendency to crystallize makes polyalcohols effective bulking agents, and this may be one of the main roles of mannitol in the basiliximab-containing drug product Simulect® (Novartis International AG, Basel, Switzerland), where it is used in combination with the cryo-/lyoprotecting excipient sucrose. Some physical properties of low molecular weight saccharides and polyalcohols are shown in Table 2.

Even though protein stability during freeze drying and subsequent storage is often dependent on the excipient/protein ratio, using higher saccharide concentrations does not always result in better protein recovery after rehydration. For example, a minimum effective disaccharide/protein molar ratio of 360/1 was determined for a freeze-dried monoclonal antibody, and increasing the excipient concentration above this no longer improved protein stability (Cleland et al., 2001). Also, utilizing a combination of two protein-protecting excipients, such as a disaccharide and polyethylene glycol (PEG), may result in better retention of the native protein structure during freeze drying than by using only the disaccharide (Prestrelski et al., 1993b).

Table 2. *Physicochemical properties of some low molecular weight saccharides and polyalcohols that have been studied as freeze drying excipients. Data gathered from publication II, Franks (2007), Kim et al. (1998), Lopes Jesus et al. (2010), Piedmonte et al. (2007), Roos (1997), Taylor and Zografi (1998) and from unpublished results. It should be noted that even thought all T_g and T_g' values have been measured with DSC, the measurement methods may vary.*

Name	Class	T_g	T_g'	Reducing/non-reducing*
cellobiose	disaccharide	108°C	-30°C	reducing
erythritol	polyalcohol	-44°C	N.A.**	non-reducing
glucose	monosaccharide	39°C	-43°C	reducing
isomalt	polyalcohol	63°C	-33°C	non-reducing
isomaltulose	disaccharide	31°C	-35°C	reducing
lactitol	polyalcohol	65°C	-32°C	non-reducing
lactose	disaccharide	114°C	-28°C	reducing
lactulose	disaccharide	79°C	-31°C	reducing
maltitol	polyalcohol	15°C	-35°C	non-reducing
maltose	disaccharide	95°C	-29°C	reducing
mannitol	polyalcohol	13°C	-30°C	non-reducing
melibiose	disaccharide	100°C	-30°C	reducing
raffinose	trisaccharide	109°C	-26°C	non-reducing
sorbitol	polyalcohol	-3°C	-45°C	non-reducing
sucrose	disaccharide	74°C	-32°C	non-reducing
trehalose	disaccharide	119°C	-28°C	non-reducing

** here a compound is considered reducing, if it participates in Maillard reaction with an amino acid with a rate that may be pharmaceutically relevant.*

*** T_g' of erythritol was not found in literature and could not be measured due to its high crystallization tendency from aqueous solutions during freezing.*

The use of reducing sugars in protein pharmaceuticals is commonly discouraged (Wang, 1999), which is one of the reasons why sucrose and trehalose, both non-reducing by

nature, are the most commonly used disaccharides in such products. Reducing sugars contain a free hemiacetal or hemiketal group, which means that the molecule coexists as both closed ring and open chain forms (Martins et al., 2001), as shown in Figure 11. The open chain form contains a reducing aldehyde or ketone group, which may participate in Maillard reaction, a form of non-enzymatic browning, by reacting with the free amino groups of proteins, usually in lysine or in a terminal amino acid, to form an N-substituted glycosilamine intermediate molecule. This can then take part in a number of chemical reactions via several reaction pathways, that ultimately lead to the formation of melanoidin co-polymers and brown nitrogenous polymers (hence the name non-enzymatic browning). In the case of mAbs, Maillard reaction can lead to the loss of positive charges on protein surfaces and the formation of acidic species (Wang et al., 2007). The reaction can take place in both aqueous and dried solid protein formulations, but the reaction kinetics generally appear to be fastest in moist amorphous mixtures with a_w around 0.4 – 0.6, which are stored above their T_g (Bell, 2007). On the other hand, the rate of Maillard reaction may be reduced by lowering the pH of the formulation, because the reactive open chain form of the sugar and the unprotonated form of the amino group are favored at higher pH (Martins et al., 2001).

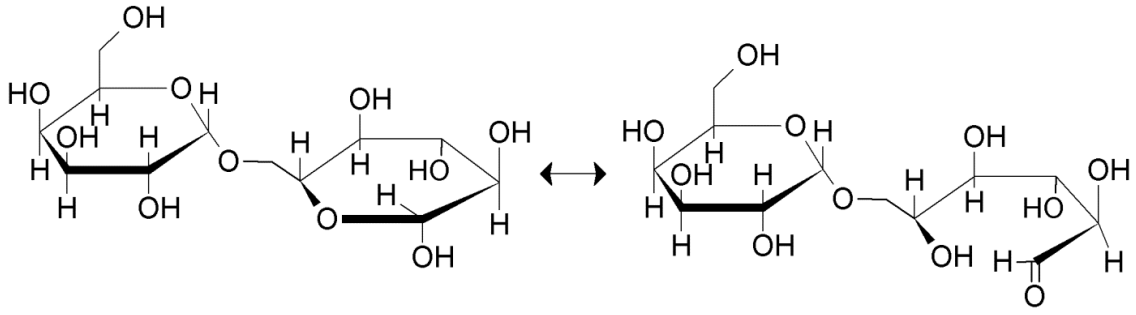


Figure 11. *The conversion between cyclic (left) and open-chain (right) forms of the glucose-monomer of melibiose (6-O-α-D-galactopyranosyl-D-glucopyranose), a reducing disaccharide. Anomeric conversion between α- and β-melibiose is not shown.*

2.5.2 Surfactants

Non-ionic surface active agents (surfactants) are sometimes used in freeze drying to reduce protein denaturation on ice/water interface (see chapter 2.3.2). Because they contain both polar and non-polar moieties, surfactants tend to concentrate on such interfaces and prohibit protein interaction with the denaturating surface (Liu et al., 2005). For this reason they can be effective as cryoprotectants, but may possess limited lyoprotecting properties (Luthra et al., 2007), possibly due to relatively poor hydrogen bond and glass forming properties. Most commonly used surfactants include polysorbate (PS) 20 and 80 (Kerwin, 2008).

2.5.3 Salts and buffers

Salts such as NaCl or KCl may be included in formulations to assure that the solution becomes as isotonic as possible upon reconstitution. This is not the only application for salts, however, as they can be used for other purposes as well. For example, NaCl has been shown to decrease the aggregation of freeze-dried tetanus toxoid during storage when compared to excipient-free samples (Schwendeman et al., 1995). Because of its high crystallization tendency, NaCl could also theoretically be employed as a bulking agent to improve the

mechanical stability of lyophilizates (Shalaev and Franks, 1996), even though this is not a common formulation strategy.

As explained in chapter 2.3.1, formulation pH is an important factor when determining the optimal stability conditions for a protein pharmaceutical. The selection of buffering compounds should be rationalized, however, as some commonly used buffers may crystallize during freeze-concentration, leading to pH shifts as explained in chapter 2.3.2 (Bhatnagar et al., 2007). Since solute crystallization is normally caused by supersaturation, an obvious solution is to use as low buffer concentrations as possible (Kasper and Friess, 2011), but this may not solve the problem for all buffering compounds. For example, disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$) often crystallizes due to freeze-concentration when sodium phosphate buffers are frozen (Gomez et al., 2001). The resulting pH shift may be significant, and a decrease of even 2 units in 8 mM phosphate buffer solutions during freezing is possible. $Na_2HPO_4 \cdot 12H_2O$ crystallization can also occur in even more dilute solutions than this, as crystalline disodium hydrogen phosphate dodecahydrate has been detected in 1 mM phosphate buffer solutions after freezing to -50°C (Varshney et al., 2006). What makes the detection of such buffer crystallization difficult is that the freeze drying end product may be totally amorphous. This is because even though $Na_2HPO_4 \cdot 12H_2O$ tends to crystallize during freezing, the dehydration of the dodecahydrate during primary drying may result in amorphicization of the crystals (Pyne et al., 2003). Nonetheless, the pH shift experienced during freezing may still be detrimental for protein stability. Other buffers that do not crystallize as easily, such as those containing citrate ions (Chang and Randall, 1992), may be more suitable for formulations which are frozen at some point during production or storage. Despite this, sodium phosphate buffers are still sometimes used in freeze-dried formulations, such as in the case of infliximab-containing product Remicade® (Janssen Biotech Inc., Horsham, PA, USA).

2.5.4 The need for new excipients for protein formulations

The number of excipients that are commonly used in freeze-dried protein-structured drug products is relatively small. Furthermore, some of those commonly used are problematic due to their seemingly sporadic behaviour during freezing, such as is the case with mannitol (Kim et al., 1998) and sodium phosphate buffers (Gomez et al., 2001). As the previous chapters have stressed, protein formulations tend to be highly unstable systems and their stabilization may be difficult. Therefore, increasing the number of excipients that could be employed in such formulations might make the work of formulation scientists easier. The problem regarding the use of new compounds as pharmaceutical excipients is that comprehensive toxicity studies would have to be carried out before a marketing authorization for a product containing them could be obtained. These are very expensive to perform, which means that only very promising compounds should be studied in clinical trials. In a highly unappealing scenario, a potential new excipient would be taken through toxicity studies only to find that there already was an excipient on the market which performs better. On the other hand, carrying out comprehensive *in vitro* feasibility studies for potential new excipients where their protein-protecting efficacy would have been proven before initiating clinical trials would reduce the risk of this kind of disaster. For this reason, this thesis focuses on comparing the efficacies of potential freeze drying excipients, some of which have not yet been tested in clinical toxicity trials.

3 Aims of the study

The aim of the thesis project was to study the mechanisms affecting the performance of disaccharides and polyalcohols as protein-protecting excipients during freeze drying and the subsequent storage. Since a given compound may not stabilize different proteins with comparable efficacy, three different model proteins were employed during the course of these studies. In addition to being studied in protein-containing formulations, the behavior of some of the excipients was explored in pure amorphous form, so that the physical properties of the protein-protecting compounds themselves could be compared.

More specifically, the aims of the present study were to:

- Show that structurally dissimilar disaccharides and polyalcohols exhibit different efficacies in protecting β-galactosidase from degradation during freeze drying and the subsequent storage at an elevated temperature, and that these efficacies can be predicted to some extent based on their molecular structures and physical properties (I,II)

- Demonstrate that the physical stability of structurally comparable disaccharides may vary during storage at different relative humidity atmospheres, and that the molecular mobility and crystallization tendency of a given excipient can affect its protein stabilization performance (I,II)

- Show how the interplay of freeze drying parameters and formulation composition may sometimes affect the stability of polyclonal IgG during drying (III)

- Prove that the efficacy of a reducing disaccharide (melibiose) in protecting rituximab from degradation can in some cases be equal to or better than that of a non-reducing disaccharide (trehalose) during freeze drying and the subsequent storage at different relative humidity atmospheres (IV)

4 Experimental

4.1 Materials, formulations and protein purification (I-IV)

The cryo-/lyoprotecting excipients used in the studies are given in Table 3. β-D-galactosidase (I) from *Aspergillus oryzae* (Mw ≈ 125 kDa, activity NLT 76.5 U/mg) was a kind gift from Amano Enzyme Inc. (Nagoya, Japan), and the protein was purified by using tangential flow filtration (TFF). Pall Minimate TFF system (Pall Co., NY, USA) with Omega 30 K molecular weight cutoff (MWCO) filter was employed with continuous filtration method using purified water as the filtration medium and by collecting 7 diafiltration volumes. After filtration the protein solution was diluted to 0.5 mg/ml concentration with purified water, and excipients (Table 3) were added to produce 1 mg/ml, 10 mg/ml and 20 mg/ml solutions (corresponding to 730:1, 7300:1 and 15000:1 excipient/protein molar ratios, respectively). The solutions were freeze dried according to the cycle given in chapter 4.2.1.

Table 3. *The names and types (R = reducing, NR = non-reducing) of the cryo-/lyoprotecting excipients used in the studies, along with the purities of the raw materials (as given by the suppliers), suppliers and trade names (if available). Furthermore, the individual studies where the excipients in question were used are expressed using the roman numerals I – IV.*

Name	Type	Purity	Supplier / Trade name	Used in
cellobiose	disaccharide (R)	> 98 %	Sigma-Aldrich Co.	(I,II)
erythritol	polyalcohol (NR)	> 99 %	Cargill Inc. / C*Eridex	(I)
isomalt	polyalcohol (NR)	> 99 %	Palatinit GmbH / galenIQ 720	(I)
isomaltulose	disaccharide (R)	> 99 %	Cargill Inc. / Xtend	(I)
lactitol	polyalcohol (NR)	> 99 %	Danisco A/S / Lactitol MC	(I)
lactulose	disaccharide (R)	> 98%	Sigma-Aldrich Co.	(I)
maltitol	polyalcohol (NR)	> 76%*	Cargill Inc. / C*Pharm Maltidex	(I)
mannitol	polyalcohol (NR)	> 99 %	Cargill Inc. / C*Pharm Mannidex	(I)
melibiose	disaccharide (R)	> 98 %	Sigma-Aldrich Co.	(I,II, IV)
sucrose	disaccharide (NR)	> 99 %	Sigma-Aldrich Co.	(I,II)
trehalose	disaccharide (NR)	> 99 %	Sigma-Aldrich Co.	(I,II, III, IV)

** maltitol was supplied as an aqueous syrup containing >76% maltitol, as well as < 2.5% sorbitol and < 0.2% reducing sugars as impurities. The syrup was dried in vacuum oven before use.*

Polyclonal bovine serum IgG (III) was obtained from Innovative Research Inc. (MI, USA), and the freeze-dried formulation consisted of 1 mg/ml IgG, 5 mg/ml trehalose, 0.01 mg/ml bovine serum albumin (BSA) and 15 mM Nacl in 1 mM Na-phosphate buffer (pH 7.4). In order to test the effects of individual excipients on IgG stability, the formulation was fractionated using size exclusion columns containing cross-linked dextran beads (HiTrap® Desalting column, GE Healthcare Biosciences Corp., NJ, USA) to remove the other excipients besides BSA. After fractionation, trehalose, NaCl and/or Na-phosphate buffer were added to create new formulations. The solutions were filtered through 0.2 µm polyethersulfone (PES) syringe filter (Acrodisc Supor®, Pall Corp., NY, USA) and 1 ml of the formulations were added

into 10 ml blow-molded vials, where they were freeze dried according to the cycle given below.

Rituximab (IV) (MabThera®, F. Hoffmann – La Roche Ltd., Basel, Switzerland) was kindly donated by the Helsinki University Central Hospital. In addition to 10 mg/ml of rituximab, MabThera® also contains 7.35 mg/ml sodium citrate, 9 mg/ml sodium chloride and 0.7 mg/ml PS80 as excipients. The removal of the original formulation excipients was attempted by fractionating MabThera® with PD-10 size-exclusion columns (Sigma-Aldrich Co., St. Louis, MO, USA) by using 10 mM citrate buffer (pH 6.3) as the eluent. The effectiveness of the fractionation protocol in removing PS80 from the protein solution was measured using the RP-HPLC quantitation method developed by Adamo et al. (2010). The protein concentration of the filtrate was measured with a UV-spectrophotometer at 280 nm by using an extinction coefficient of 1.49 ml mg^{-1} cm^{-1} (Hawe et al., 2010). The rituximab solutions were diluted to 1 mg/ml concentration with 10 mM citrate buffer (pH 6.3) and trehalose or melibiose was added to produce two formulations with 1 mg/ml rituximab, 10 mM citrate buffer and 50 mg/ml of either trehalose or melibiose. The solutions were freeze dried according to the cycles given below.

4.2 Methods

4.2.1 Freeze drying (I-IV)

β-galactosidase (I), polyclonal bovine serum IgG (III) and rituximab (IV) solutions were freeze dried using Lyostar II lyophilizer (SP Industries Inc., Warminster, USA). Pure disaccharide samples (II) were pre-frozen in two different freezers at -20°C and -80°C for 24 h each, and then freeze dried using Lyovac GT2 freeze drier (Amsco Finn-Aqua GmbH, Germany) for 72 h at 150 mT. The resulting lyophilizates were further equilibrated under vacuum in desiccators containing phosphorous pentoxide (P_2O_5) for one week to further reduce their residual water contents before initiating the storage experiments. The freeze drying cycles used in different studies are given in Table 4. In study (III) the primary drying pressure and secondary drying heating rate were altered between three different processing values, denoted by numbers -1, 0 and 1. In studies (I) and (III) the sample temperatures were measured during drying with thermocouples, which were inserted into the vials so that they were in direct contact with the solutions being freeze dried. The drying chamber was filled with dry N_2 after each freeze drying cycle, and the vials closed by either compressing the vials inside the chamber (I, III), or by fixing the stoppers immediately after sample removal (II, IV).

4.2.2 Sample storage

β-galactosidase lyophilizates (I) were rehydrated with purified water and analyzed after freeze drying, or after being stored for 7, 30 or 90 days in closed vials at 45°C. This storage temperature was used, because it has been employed earlier to bring out the differences in storage stabilities of different β-galactosidase strains (Palumbo et al., 1995). Pure disaccharide lyophilizates (II) were stored in open vials at ambient temperature in desiccators containing different relative humidity atmospheres (RH 0%, 11%, 23%, 33% and 43%) for up to 46 days and analyzed periodically. Higher relative humidity atmospheres, e.g. RH 60% as is suggested by the ICH guideline Q1A-R2 (ICH, 2003), were not used, because lyophilizate collapse occurred in all samples at RH 43% or lower, and studying samples well

above their collapse point was not considered relevant in these experiments. Polyclonal bovine serum IgG lyophilizates (III) were analyzed after freeze drying without subjecting them to a specific storage period. Rituximab lyophilizates (IV) were rehydrated with 0.22 µm filtered purified water and analyzed after freeze drying, or after being stored for 30, 60 or 90 days at 25°C in loosely stoppered vials to allow moisture sorption to occur in desiccators containing different relative humidity atmospheres (RH 5%, 11% and 23%). Storage conditions mimicking room temperature (i.e. 25°C) were used instead of those representing refridgerated conditions (i.e. 2-8°C) in order to increase the rate of protein degradation reactions.

Table 4. *The freeze drying cycles used in different studies, where shelf temperatures (T_{shelf}), stage times (t) and chamber pressures (P) of different drying stages were altered according to the scheme shown in the table. Study (II) was carried out using a drier without T_{shelf} control.*

Study	Freezing	Primary drying	Secondary drying
	T_{shelf} ; t	T_{shelf} ; t ; P	T_{shelf} ; (Δ)t ; P
(I)	1) 5°C ; 0.5 h 2) −45°C ; 2 h 3) −40°C ; 0.5 h	−35°C ;21 h ; 150 mT	1) → 35°C at 5°C / h ; 150 mT 2) 35°C ; 2 h ; 150 mT
(II)	1) -20°C ; 24 h 2) -80°C ; 24h	- ; 72 h ; 150 mT	-
(III)	−40°C ; 3.5 h	-1: −25°C ; 21 h ; 20 mT 0: −25°C ; 21 h ; 60 mT 1: −25°C ; 21 h ; 100 mT	-1: → 40°C at 5°C / h ;60 mT 0: → 40°C at 30°C / h ;60 mT 1: → 40°C at 60°C / h ;60 mT (total sec. drying time always 16 h)
(IV)	1) 5°C ; 0.5 h 2) −45°C ; 3 h	−40°C ; 25 h ; 100 mT	→ 20°C at 2.5°C / h ; 100 mT

4.2.3 Protein in vitro *activity and binding analysis (I, III)*

β-galactosidase activity (I) after freeze drying and storage was measured with spectrophotometric o-nitrophenyl-β-D-galactopyranoside (ONPG) cleavage rate test (n = 3) at 420 nm (Sambrook et al., 1989). The results were compared to a standard curve, and the β-galactosidase activity after TFF purification was assigned as 100% in relative activity. The purity of the filtered β-galactosidase solution was not examined, because for the sake of comparing the protein-protecting efficacies of different excipients, it was not necessary to fully characterize the filtrate. Polyclonal bovine serum IgG (III) *in vitro* binding activity was measured with enzyme-linked immunosorbent assay (ELISA) in 96-well plates using a commercial polyclonal ELISA test (Bethyl Laboratories Inc., TX, USA). The plates were coated with bovine IgG, washed and then blocked with PS20. Lyophilizates (n = 3) were rehydrated with 1 ml purified water, equilibrated for 60 min and diluted before incubation on the coated well plates. After incubation, the wells were washed, incubated with horseradish peroxidase -conjugated IgG, washed again and finally incubated using tetramethylbenzidine solution. The reaction was stopped using dilute sulfuric acid solution and the absorbance

measured at 450 nm using an automated well plate reader (Varioskan 3001, Thermo Fisher Scientific, MA, USA). The results were compared to a standard curve, which was generated separately on each well plate from a reference bovine IgG solution. All well plate pipetting and washing was carried out by using an automated work station (TECAN Genesis RPS 8/150, Tecan Group Ltd., Switzerland) in order to minimize the effect of pipetting errors.

4.2.4 Lyophilizate solid state analysis (I-IV)

Lyophilizate T_g and aqueous formulation T_g' values were measured by using differential scanning calorimetry (DSC) with DSC823e (Mettler Toledo Inc., Zürich, Switzerland) (I - IV). The samples enclosed in hermetically sealed 40 µl aluminum pans, equilibrated at a given temperature for 3-10 min and heated at 10°C/min under 50 ml/min dry N_2 flow to a given temperature. T_g and T_g' values were taken as transition midpoints.

Lyophilizate crystallinity was studied with x-ray powder diffractometry (XRPD) by using Bruker D8 Advance (Bruker AXS Inc., WI, USA) (I, II, IV). The samples were scanned along a given 2θ range at 0.05° - 0.1°/s rate. If a crystal diffractogram was detected, it was compared to calculated diffractograms acquired from Cambridge Structural Database (CSD) by using ConQuest 1.10 program (Cambridge Crystallographic Data Centre, Cambridge, UK) in order to identify the polymorph(s) in question.

Lyophilizate water contents were measured with two different methods. Volumetric Karl-Fisher titration (KF) with MT-V30 (Mettler Toledo, Switzerland) was used to analyze the larger lyophilizates (II, IV), and thermogravimetric analysis (TGA) with TGA 850 (Mettler-Toledo Inc., Switzerland) was used in the case of smaller samples (I, III). In KF measurements the lyophilizates were ground in a glovebox under low relative humidity (≈ 5%) before transferring them to the measurement cell in order to increase sample dissolution rate. TGA measurements were carried out by weighing 5-10 mg of sample into 70 µl alumina crucibles, equilibrating it at 25°C for 5 min and then heating at 20°C/min to 120-130°C under 50 ml/min dry N_2 flow, where the sample was again equilibrated for 5 min. The water contents were calculated from the resulting weight losses.

Lyophilizate water sorption rates were studied by using two different methods. In desiccator measurements the water contents were determined gravimetrically (n = 4), after having measured the empty vial masses, as well as the initial lyophilizate water contents by using KF. In addition, isothermal microcalorimetry (IMC) was performed with TAM 2277 (Thermometric AB, Sweden). The IMC included a sequential RH-unit setup, where a gas flow with controlled relative humidity was first directed to the calorimeter cell containing the sample (n = 1). The gas flux exiting the sample cell was then lead to a second cell, where it was moistened to RH 100% while measuring the moisturization heat. As a result, the sample water sorption and desorption could be observed in addition to other thermodynamic transitions occurring in the sample. The ground lyophilizate samples were ramped from RH 0% to RH 60-70% at 25°C by 10% increases every 4 h, while continuously measuring changes in heat flow.

4.2.5 Sample visualization (II)

In order to study whether viscous flow had occurred in amorphous lyophilizates during processing and storage, pure disaccharide samples were analyzed with scanning electron microscopy (SEM). The samples were coated with a thin platinum layer in Quorum Q150TS sputter (Quorum technologies Inc., Ontario, Canada) and scanned using FEI Quanta 250

FEG (FEI Inc., OR, USA) microscope under 60 Pa pressure with large field vacuum secondary electron detector.

4.2.6 Molecular mobility measurement (II)

Relaxation time constants relating to the molecular mobility of freeze-dried trehalose and melibiose lyophilizates were measured after their water contents had been equilibrated to a given level. Solid-state NMR (ssNRM) cross polarization magic angle spinning (CPMAS) experiments were carried out using Bruker Avance III 500 spectrometer (Bruker BioSpin GmbH, Ettlingen, Germany) with magnetic flux density of 11.7 T, and with 4 mm double resonance broad band VTN CPMAS probehead. Samples (n = 1) were packed into ZrO_2 rotors plugged with KEL-F endcaps, and the sample spinning rate was set to 10 kHz. The longitudinal relaxation time constants of protons (T_1H) were measured at 35°C by using a saturation recovery experiment with cross polarization step and carbon detection. Contact time for cross polarization was 500 μs, and the signal acquisition time was 10 ms, during which proton decoupling (SPINAL-64) with a rf-field strength of 63 kHz was used. The spectra were referenced externally via adamantane by setting the low field resonance at 38.48 ppm. The intensity of the largest signal that was found in the spectrum close to 72 ppm for trehalose and 70 ppm for melibiose was measured at eight delay times after the saturation step. An exponential fit of the obtained data was carried out by using TopSpin 2.1 software (Bruker BioSpin GmbH, Ettlingen, Germany).

4.2.7 Protein secondary structure analysis (I, III, IV)

Fourier transform infrared (FT-IR) spectroscopy was used to study β-galactosidase secondary structure directly from the lyophilizates (I). 1-2 mg of the lyophilizate (n = 5) was ground with ≈ 250 mg of FT-IR grade KBr (221864, Sigma-Aldrich, MO, USA) and compressed into a pellet under vacuum. The samples were analyzed using Vertex 70 (Bruker Optics Inc., MA, USA) FT-IR spectrometer by measuring the spectra 20 times with 4 cm^{-1} resolution between 4000-650 cm^{-1} under dry air flow. The spectra were further treated using OPUS 4.0 program (Bruker Optics Inc., MA, USA) by calculating their second derivatives, after which they were smoothed with Savitzky-Golay function by using a convolution width of 9 points. The resulting spectra were baseline corrected along 1720- 1490 cm^{-1} region (amide I and II regions) to minimize the effect of differing baselines on further data analysis.

Far-UV circular dichroism (CD) spectra of rehydrated samples (n = 1-2) were measured using Jasco J-720 (III) or J-815 (IV) CD spectrometers, which used Jasco PTC-4235 units to control the measurement temperature (Jasco International Ltd., Tokyo, Japan). The samples were diluted to 0.1 mg/ml protein concentration and the spectra were measured from a 1 mm path length quartz cuvette in the far-UV region at 25°C temperature. The scan rate varied between 12 - 200 nm/min and each final spectrum was averaged from 5-6 consecutive scans. The resulting spectra were background corrected by subtracting the corresponding blank buffer spectrum from the result, and either used as such (III) or after being smoothed with Savitzky-Golay function by using a convolution width of 25 points (IV). Finally, the CD signals were converted to mean residual ellipticity (MRE) according to Kelly et al. (2005).

4.2.8 Protein aggregation, fragmentation and monomer recovery analysis (III, IV)

The concentrations and size distribution of particles with a diameter between 1 – 100 μm (IV) were measured from rehydrated solutions with light obscuration (LO) by using PAMAS SVSSC with HCB-LD-25/25 sensor (Partikelmess und Analysesysteme GmbH,

Rutesheim, Germany). Samples (n = 3) were measured three times with a sample volume of 0.2 ml per measurement, using emptying and rinsing rates of 10 ml/min. Prior to each measurement the system was flushed with 0.22 µm syringe-filtered purified water until particle counts of less than 50 particles/ml ≥1 µm and less than 10 particles/ml ≥10 µm were reached.

Dynamic light scattering (DLS) measurements were performed with a Malvern Zetasizer 3000 HS (III) or Zetasizer Nano ZS (IV) (Malvern Instruments Ltd., Worcestershire, UK). The measurements (n = 3-6) were carried out from rehydrated solutions at 25°C in single-use polystyrene cuvettes (Fisher Emergo B.V., Landsmeer, the Netherlands) with a path length of 10 mm. 3x10 runs of 60 seconds each (III) or 3x15 runs of 10 seconds each (IV) were performed, and the average intensity size distributions, Z-average diameters (Z-ave) and polydispersity indexes (PDI) of the formulations were obtained.

Nanoparticle tracking analysis (NTA) of the particles with a diameter between 50 – 1000 nm (IV) was performed by using NanoSight LM20 (NanoSight Ltd., Amesbury, UK), equipped with a 640 nm laser and a Viton fluoroelastomer O-ring. The benefits and limitations of NTA as a counting and sizing method for protein aggregates has been discussed in detail elsewhere (Filipe et al., 2010a). The particle counts and size distributions of rehydrated samples (n = 2) were analyzed with three 40 second measurements. Detection threshold was varied between 5 – 20 and gain between 0.5 – 3 depending on the particle content of samples. When necessary, samples were diluted to limit the maximum number of particles per images to ≈ 50. Minimum particle size was set to 50 nm to exclude potential error signals caused by large rotating irregular particles. Prior to each measurement the system was flushed with 0.22 µm syringe-filtered purified water. The mean particle sizes and particle concentrations were calculated with NTA 2.0 software (NanoSight Ltd., Amesbury, UK).

The changes in soluble polyclonal bovine IgG monomer contents (III) were studied with asymmetric flow field flow fractionation (AF4). AF2000 (Postnova Analytics, Lansberg, Germany) model AF4 was connected to UV detector (PN3211, Postnova Analytics, Lansberg, Germany) at 280 nm wavelength for protein concentration analysis. Lyophilizates were rehydrated using 1 ml of purified water and equilibrated at room temperature for 60 min before injection. The eluent (8.5 mM phosphate buffer with 150 mM NaCl and 0.02%-w/w NaN_3, at pH 7.4) and the sample solution (protein concentration 1 mg/ml, injection volume 50 µl, n = 3) were delivered to the inlet of the AF4 channel at the rate of 0.2 ml/min. Focusing time was 4 minutes, during which the focusing flow was 4.8 ml/min, the cross flow was 4.0 ml/min while the rest of the eluent (at 1.0 ml/min) exited through the channel outlet towards the detectors in order to make the solution pass continuously through the detectors. The transition time from injection/focusing to elution was 1.0 min, and during elution the focusing flow was stopped and eluent flow rate increased to 5.0 ml/min. The channel outlet and the cross flow rates were 1.0 and 4.0 ml/min, respectively. Elution stage lasted 40-90 minutes depending on the sample, and the resulting data was further treated using NovaFFF software (Postnova Analytics, Lansberg, Germany). Soluble IgG monomer contents were calculated by comparing the maximum monomer UV signal peak height of the native samples to those of the freeze-dried samples.

High-performance size-exclusion chromatography (HP-SEC) of rehydrated rituximab samples (IV) was performed on an Agilent 1200 chromatography system, that was equipped with UV and fluorescence detectors (Agilent Technologies, Palo Alto, USA), as well as DAWN® HELEOS™ multi angle laser light scattering (MALLS) detector (Wyatt Technology Europe GmbH, Dernbach, Germany). The running buffer was composed of 100 mM

phosphate buffer (pH 7.1), 100 mM Na_2SO_4 and 0.02%-w/w NaN_3. Sample injection volume was 50 µl with 0.5 mg/ml protein concentration, and separation was carried out at 0.5 ml/min for 40 minutes using a TSKgel 3000 SW_{XL} 7.8 mm x 300 mm column with a TSKgel 4000 SW_{XL} guard column (Tosoh Bioscience Corp., Tokyo, Japan). Samples (n = 3) were centrifuged at 8000 rpm for 5 min before injection to protect the columns from large insoluble aggregates. UV detection was performed at 280 nm, and soluble rituximab monomer content was calculated by comparing the area under the curve (AUC) of the monomer peak in the freeze-dried samples to that of the unstressed one (which was set at 100%). Similar method has been used by other studies, where protein monomer contents before and after processing has been compared (Cleland et al., 2001; Hawe et al., 2009, Schersch et al., 2010). Fluorescence excitation was carried out at 280 nm and emission was measured at 350 nm. The light scattering data was used to calculate protein molar masses at different retention times by using Astra V software (Wyatt Technology Europe GmbH, Dernbach, Germany).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of rehydrated samples (IV) was carried out using Mini-PROTEAN® tetra cell gel electrophoresis system (Bio-Rad Laboratories Inc., Hercules, CA, USA). Samples that were pretreated under reducing (with β-mercaptoethanol) and non-reducing conditions (n = 1), were ran on Bio-Rad Mini-PROTEAN TGX 4-15% tris-glycine precast gels along with Bio-Rad prestained broad range SDS-PAGE standards (Mw-range ≈ 6 – 200 kDa). The gels were run for approximately 30 minutes, and after electrophoresis the gels were stained for 15 minutes with Coomassie Brilliant Blue (Bio-Rad Laboratories Inc., Hercules, CA, USA), and destained overnight with 10% V/V methanol, 10 % V/V glacial acetic acid and 80% V/V water solution. Finally, the destained gels were scanned and the molecular weights of different protein species were calculated based on the standard lanes.

4.2.9 Multivariate and statistical analysis (I, III)

Multivariate data analysis (partial least squares, PLS) of the β-galactosidase FT-IR spectra (I) was carried out using Simca-P® 10.5 program (Umetrics AB, Umeå, Sweden). Differences between spectra were visualized by plotting their principal components (PC) on a coordinate system, resulting in a scope plot where each PC was represented by a different axis. Therefore, two identical spectra with identical PCs would be superimposed on the score plot. The PLS model was also be used to identify which sections of the spectra had the greatest impact on different PCs using the loadings plot. In the case of FT-IR spectra, the wave number regions which underwent significant changes displayed large loading values. This allowed the loadings plots to be used in conjunction with the score plots for the identification and interpretation of spectral changes from very large amounts of data.

Polyclonal IgG freeze drying experimental design and data analysis (III) was carried out by using Modde® 7.0 software (Umetrics AB, Umeå, Sweden). A randomized face-centered central composite design (CCF) setup was created, where two factors, primary drying pressure (Pprim) and secondary drying heating rate (ΔTsec), were varied on three levels (Table 4), and by evaluating cycle-to-cycle variation from three midpoint repeats (Pprim = 60 mTorr, ΔTsec = 30°C/h). Six analytical responses (see chapter 5.4) were employed in the characterization of protein stability, and a PLS model was formed to evaluate the statistical significance of each response with 95% confidence level. The models were evaluated based on the goodness of fit (R^2) and goodness of prediction (Q^2) values of different responses. R^2 represents the percentage of variation in the results explained by the model (i.e. how well the data fits the model), whereas Q^2 shows the efficacy of the model in

predicting new data (i.e. how well experimental variation is predicted). The limit of $p < 0.05$ was used when evaluating whether model could be generated where a factor contributed significantly to the variation of a response.

5 Results and discussion

5.1 General remarks

Using three different model proteins during the course of these studies served several purposes. Firstly, using only one model protein would have limited the evaluation of the cryo-/lyoprotecting properties of different excipients to that particular protein, making the generalization of the results difficult. Also, prior to these studies no published results could be found regarding the stability of rituximab during freeze drying, whereas β-galactosidase from different sources has been frequently employed in drying studies (Pikal-Cleland and Carpenter, 2001; Vasiljevic and Jelen, 2003; Yoshioka et al., 2007). Therefore it was easier to study a large number of excipients using a model protein where ample stability data was readily available. The selection of freeze drying parameters for the final stability study with rituximab was carried out with polyclonal bovine IgG, because it was relatively easy to acquire in quantities that were needed for the process optimization studies (> 1 g). Finally, using a pharmaceutically relevant protein (rituximab) was necessary to establish a proof-of-concept for the use of melibiose in further freeze drying studies of protein-structured pharmaceuticals.

In addition to many relatively poorly studied compounds, the excipient list (Table 3) also contained some well-known freeze drying excipients, such as trehalose, sucrose and mannitol. This was necessary, because one aim of this study was to establish some kind of ranking order within the group of compounds used. If such a ranking order would have been created without including certain “golden standards”, the results might have remained a curiosity with little practical value. Now, the results are intended to be more comparable with other freeze drying studies that deal with the use of excipients in improving protein stability.

5.2 Initial excipient comparison and the selection of compounds for further studies (I)

The focus of the initial study was to select a number of interesting cryo-/lyoprotective excipients for further studies. This selection was carried out by investigating how a model protein (β-galactosidase) was protected during freeze drying and storage by different compounds.

5.2.1 The enzymatic activity of β-galactosidase after freeze drying

The relative enzymatic activity of β-galactosidase remaining in different freeze-dried formulations after drying and the subsequent storage at 45°C are shown in Figure 12. It can be seen that without any cryo-/lyoprotecting excipients, protein activity was only approximately 13% after freeze drying, and no activity was detected after storing it for 7 days

or longer. This meant that the protein was highly unstable when not protected by an excipient. On the other hand, activity after freeze drying was usually > 90% when an excipient was used, except in the case of mannitol and erythritol, where it was < 50%. Most differences between excipients became visible during storage. The relative activity preservation after 90-day storage period was generally good when using disaccharide-structured excipients, and as seen from Table 5, the three highest ranking compounds were disaccharides with each excipient/protein molar ratio. Compounds such as lactitol, lactulose and maltitol displayed a clear trend, where an increased excipient/protein molar ratio led to an improvement in protein activity retention during storage, whereas altering this ratio did not have a clear effect when melibiose or trehalose were used. At 730:1 excipient/protein molar ratio, cellobiose and sucrose formulations appeared to reach a plateau in β-galactosidase activity after 30 days of storage, where further 2 months of storage did not reduce the activity significantly. In other formulations the decreasing activity trend continued throughout the whole storage period.

Excipient crystallization was most likely the cause for poor β-galactosidase stability in mannitol and erythritol formulations, where the XRPD analyses confirmed that crystallization had taken place during freeze drying (data not shown). Clear signs of crystallization were not detected in other samples after freeze drying or storage, but small sample sizes meant that crystalline regions might have always not been detected.

5.2.2 Factors contributing to freeze-dried β-galactosidase stability

Based on their protein activity preservation efficacies (Fig. 12) and physical properties (Table 2 in chapter 2.5.1), the excipients studied here could be roughly divided into three categories. The first category included polyalcohols with low T_g (< 15°C), namely mannitol and erythritol, which were not effective in protecting β-galactosidase due to their high crystallization tendency. The second category included compounds with intermediate T_g (15 - 80°C), which displayed a molar ratio -dependent inhibitory effect on activity loss. These included isomalt, isomaltulose, lactitol, lactulose, maltitol and sucrose. The third category included high T_g (> 100°C) compounds melibiose and trehalose, which appeared equally effective with all concentrations used. Despite its high T_g of 108°C (Table 2), cellobiose was not effective at 730:1 excipient/protein molar ratio, so it could not be included in the third category. Furthermore, the efficacies of isomalt and isomaltulose in upholding the enzymatic activity of β-galactosidase were not very clearly dependent on their molar ratios, but their low/intermediate T_g values meant that they could not be included in the third category.

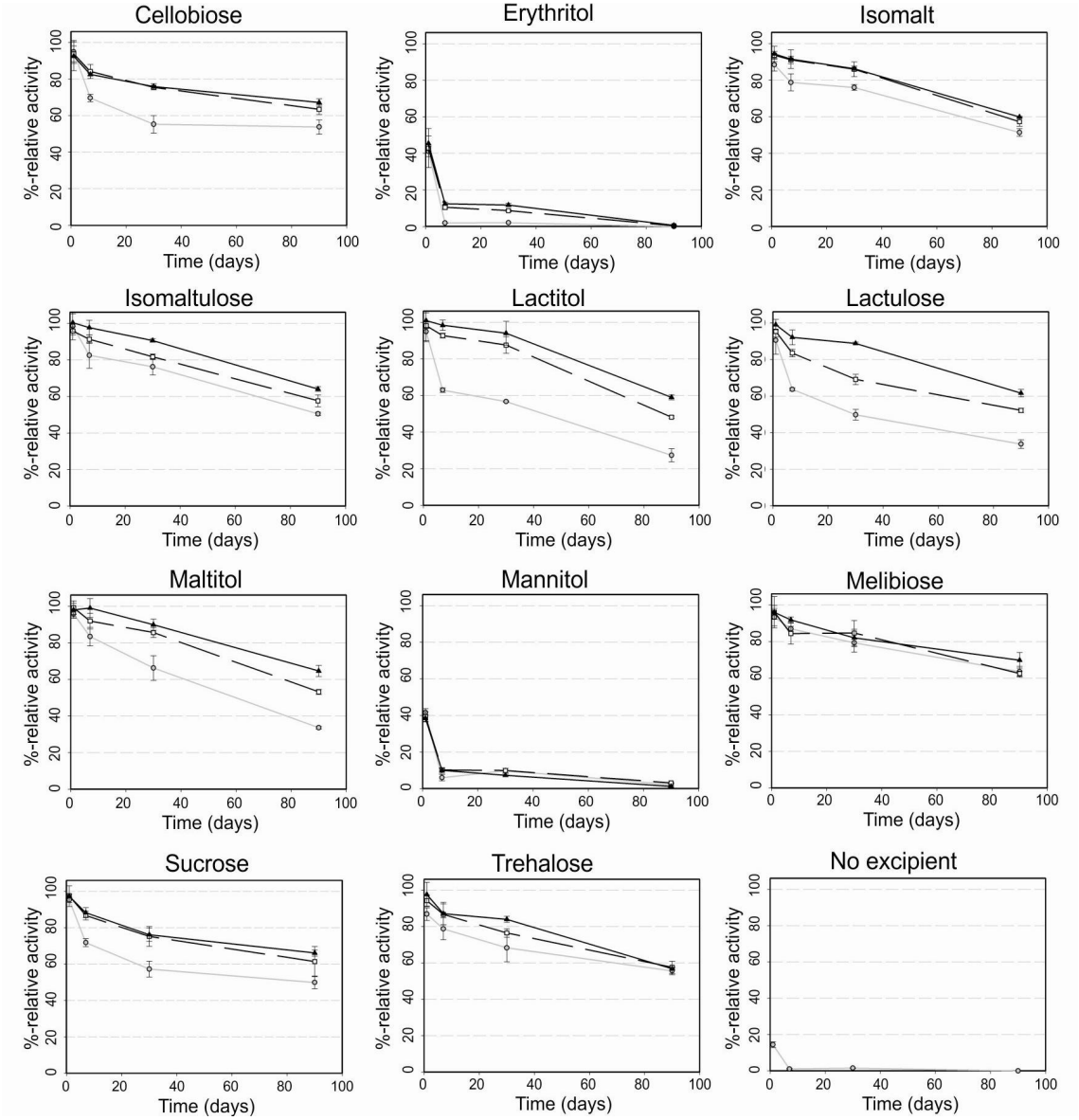


Figure 12. *Relative activity of freeze-dried β-galactosidase (concentration before freeze drying 0.5 mg/ml) during storage at 45°C. when using different excipients with 730:1 (gray line), 7300:1 (dashed line) or 15000:1 (black line) excipient/protein molar ratios before freeze drying.*

On the basis of the results, cellobiose, melibiose, sucrose and trehalose were selected for further studies. The reasons for their differing properties in inhibiting the enzymatic activity loss of β-galactosidase during storage could not be elucidated based on the data available, meaning that additional experiments were required. Furthermore, it was necessary to assess whether other protein properties besides enzymatic activity were affected by storage at elevated temperature in similar manner.

Table 5. *The rank order of relative β-galactosidase activities after 90-day storage at 45°C, when using different cryo-/lyoprotecting excipients with different excipient/protein molar ratios (E/P), with standard deviations (for samples from three different vials) given in brackets.*

Rank	E/P = 730:1		E/P = 7300:1		E/P = 15000:1	
	excipient	Rel. act. (%)	excipient	Rel. act. (%)	excipient	Rel. act. (%)
1	melibiose	64 (± 3)	sucrose	66 (± 3)	melibiose	70 (± 4)
2	trehalose	56 (± 2)	cellobiose	63 (± 3)	cellobiose	66 (± 2)
3	cellobiose	52 (± 3)	melibiose	62 (± 1)	sucrose	66 (± 2)
4	isomalt	51 (± 2)	isomaltulose	58 (± 3)	maltitol	65 (± 3)
5	isomaltulose	51 (± 1)	trehalose	58 (± 3)	isomaltulose	64 (± 1)
6	sucrose	50 (± 3)	isomalt	57 (± 2)	lactulose	62 (± 2)
7	lactulose	33 (± 2)	maltitol	53 (± 1)	isomalt	60 (± 0)
8	maltitol	33 (± 1)	lactulose	52 (± 1)	lactitol	59 (± 1)
9	lactitol	27 (± 4)	lactitol	48 (± 1)	trehalose	57 (± 1)
10	mannitol	2 (± 1)	mannitol	3 (± 0)	mannitol	1 (± 0)
11	erythritol	0 (± 0)	erythritol	0 (± 0)	erythritol	0 (± 0)

5.3 The effect of disaccharide physical properties on protein secondary structure retention during storage (I, II)

The secondary structure alterations of β-galactosidase during storage in different disaccharide formulations were investigated in order to compare whether similar changes took place during storage as indicated by the activity analyses. It was hypothesized that despite their similar molecular structures, differences in the physical properties of the four disaccharides of interest might play an important role in protein stability. Therefore, water sorption, crystallization and water plasticization tendencies of pure disaccharides in amorphous state were also studied at different relative humidity atmospheres.

5.3.1 Changes in β-galactosidase secondary structure during storage

The efficacies of trehalose, melibiose, cellobiose and sucrose (molecular structures shown in Figure 13) in inhibiting the secondary structure alterations in β-galactosidase were compared during storage at 45°C. For this purpose, lyophilizates with 730:1 excipient/protein molar ratio were analyzed using FT-IR during a storage period of 90 days. Figure 14 shows the score plot and Figure 15 the loadings plot of the three component PLS model, which was generated from the resulting spectra at 1720 – 1490 cm^{-1} region. This wave number region displays the so-called amide I and II absorption peaks, which result mainly from C=O stretching and N-H vibrations, respectively (Muga et al., 1993). It is therefore a region of interest when observing differences in the amide bond vibrations, which are related to protein secondary structure alterations.

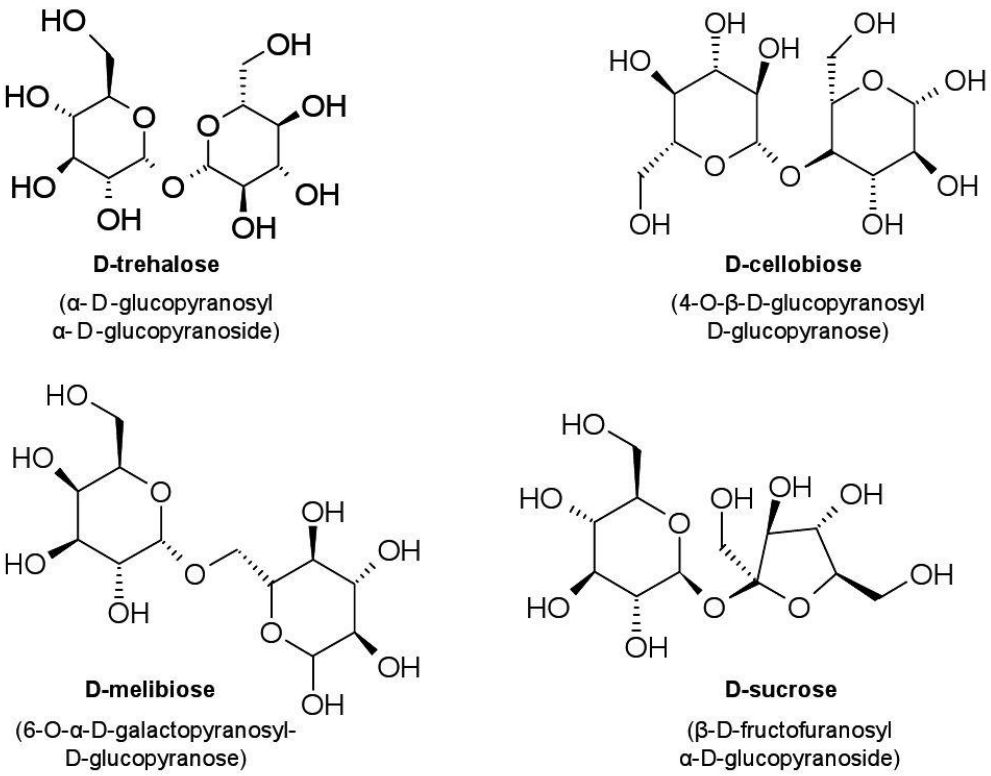


Figure 13. *The molecular structures of trehalose, cellobiose, melibiose and sucrose. Copyright © (2011) Springer Ltd., reprinted with permission.*

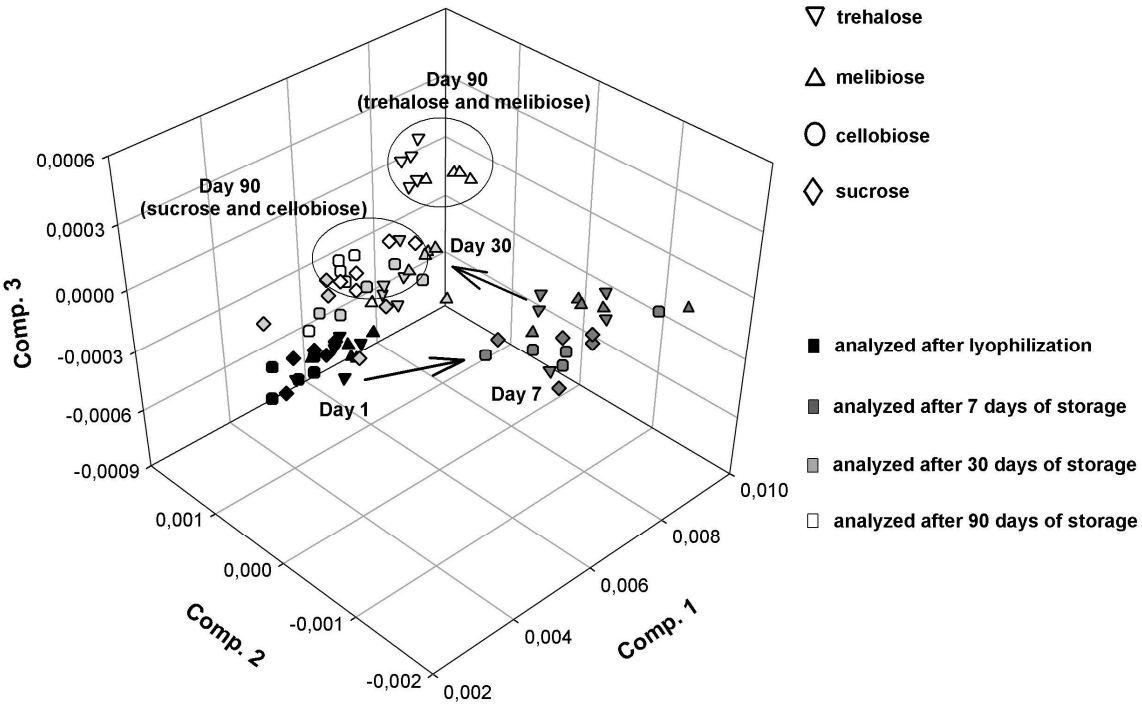


Figure 14. *PLS score plot of FT-IR spectra, which were taken during the 90-day storage period at 45°C from β-galactosidase lyophilizates (n = 5, 730:1 excipient/protein molar ratio). Copyright © (2011) Springer Ltd., reprinted with permission.*

The score plot (Fig. 14) suggests that β-galactosidase secondary structure underwent similar perturbations in all four formulations during storage up to 30 days, as the points representing the three PCs of the spectra are clustered. However, after storing the lyophilizates for 90 days the PC values of trehalose and melibiose were separated from the cluster of sucrose and cellobiose, which on the other hand remained relatively unchanged from day 30 PCs. While these results did not allow the quantitation of protein secondary structure alterations in different formulations, the FT-IR analyses did display a similar trend as the enzymatic activity measurements (Fig. 12). In those the relative activity did not decrease significantly in sucrose and cellobiose lyophilizates after storing them for 30 days at 45°C. In trehalose and melibiose lyophilizates, however, β-galactosidase activity was still decreasing after 30 days of storage.

The loadings plots (Fig. 15) were used to interpret the secondary structure alterations, which were most likely causing the changes observed in the FT-IR spectra during storage. The first significant region of variance in PC1 was found around 1640 cm^{-1}, between the absorption regions of β-sheet (≈ 1635 cm^{-1}) and α-helix (≈ 1650 cm^{-1}) structures, possibly denoting a transition from the former structure to the latter. The second location where an increase in absorption was observed was located in the α-helix region at 1650 cm^{-1}. Additionally, less clear structural alterations were also observed around β-turn (1690 – 1665 cm^{-1}), distorted β-structure (≈ 1615 cm^{-1}) and combined α-helix and β-structure (1560 – 1520 cm^{-1}) regions. The general conclusion that could be drawn from the spectra was that the portion of α-helix structures increased, possibly at the expense of β-sheet structures. Similar structural perturbations have been shown to occur in β-galactosidase when it has been thermally denatured in solutions containing SDS (Muga et al., 1993).

These results supported the enzymatic activity analyses (730:1 excipient/protein molar ratio) of β-galactosidase. After 30 days of storage, the decrease in enzymatic activity and change in secondary structure seemed to stop in sucrose and cellobiose formulations, but they both continued in trehalose and melibiose formulations. On the other hand, the enzymatic activity loss was less significant in trehalose and melibiose formulations during the first 30 days of storage. Such trend was not visible in the secondary structure analyses, but the quantitation of structural alterations was not attempted in these studies, as it may be considered unreliable in many cases (Surewicz et al., 1993). It was hypothesized that the differences in the protein-protecting efficacies of the disaccharides studied here might have caused differences in their crystallization tendencies, as similar effects were observed in the case of erythritol and mannitol. Proof regarding such differences was sought by comparing the physical stabilities of pure disaccharides under different experimental conditions.

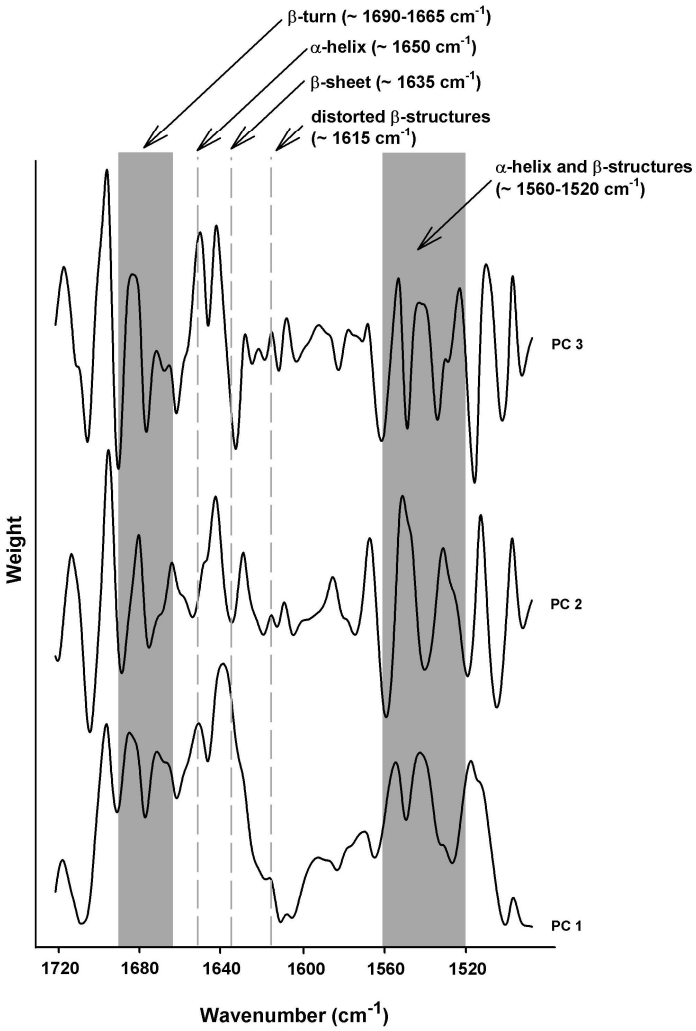


Figure 15. *PLS loadings plots of FT-IR spectra, which were taken during the 90-day storage period at 45°C from β-galactosidase lyophilizates with 730:1 excipient/protein molar ratio. Protein secondary structures absorbing at given wavenumber regions have been interpreted according to literature (Arrondo et al., 1989; Cooper and Knutson, 1995; Dong et al., 1990; Muga et al., 1993) Copyright © (2011) Springer Ltd., reprinted with permission.*

5.3.2 Water sorption and crystallization kinetics in pure amorphous disaccharides

Pure freeze-dried disaccharides were equilibrated at different relative humidity atmospheres and their water sorption/desorption rates and crystallization onset times compared as shown in Figure 16. During the 500 h storage period, crystallinity was detected in cellobiose at RH ≥ 23%, in sucrose at RH ≥ 33%, in trehalose at RH = 43% and not at all in melibiose. Still, it did appear that crystallization was initiated in trehalose and melibiose samples at RH 33% after approximately 300 h, as their water contents began to decrease, but this occurred in such small scale that it could not be detected by the XRPD. A clear melibiose monohydrate diffractogram was acquired only after the samples had been stored at RH 43% for 1100 h (data not shown). When comparing the other x-ray diffractograms to those acquired from CSD, cellobiose and sucrose were shown to crystallize as anhydrates and trehalose as partly dihydrate and partly β-anhydrate.

All lyophilizates collapsed quickly when stored at RH 43%, and in case of sucrose this also occurred at RH 33%. Furthermore, it was clear that cellobiose crystallization had

commenced after 91 h storage at RH 33%, but unlike sucrose, the lyophilizate did not collapse macroscopically. The slower crystallization rate of amorphous melibiose compared to trehalose was also verified by using IMC, where the latter exhibited almost an instantaneous crystallization at RH 60%, whereas the crystallization rate of the former was slow even at RH 70% (data not shown).

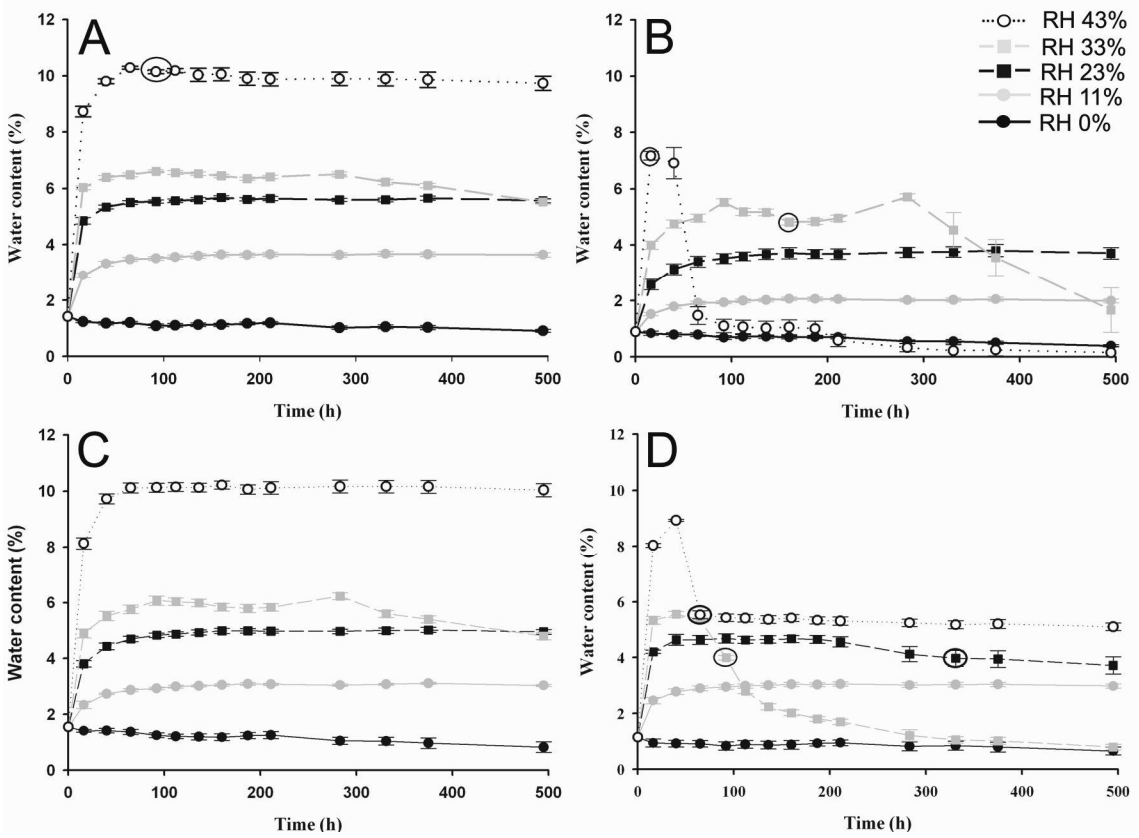


Figure 16. *The water contents (%-w/w) of trehalose (A), sucrose (B), melibiose (C) and cellobiose (D) during storage at RH 0 – 43% (n = 4). The first time points where crystalline regions were detected with XRPD are marked with circles. Copyright © (2012) Springer Ltd., reprinted with permission.*

5.3.3 The plasticizing effect of water sorption on the molecular mobility of pure disaccharides

The GT and BET model results are shown in Figure 17 and Table 6. The table also includes the critical water content values [C_{crit}(H2O)], where lyophilizate T_g was depressed to match the storage temperature (25°C). The water content values of RH 43% were not used in the BET models, as including them resulted in poor model fits. This coincided with exceeding $C_{crit}(H_2O)$ for trehalose, melibiose and cellobiose, and was relatively close in the case of sucrose, implying that lyophilizate collapse resulted in a change in its water sorption behavior. Furthermore, the results at RH 43% could not be reliably used to model the sorption tendencies, as all disaccharides discounting melibiose started to crystallize relatively quickly under those conditions, which tended to affect their water contents.

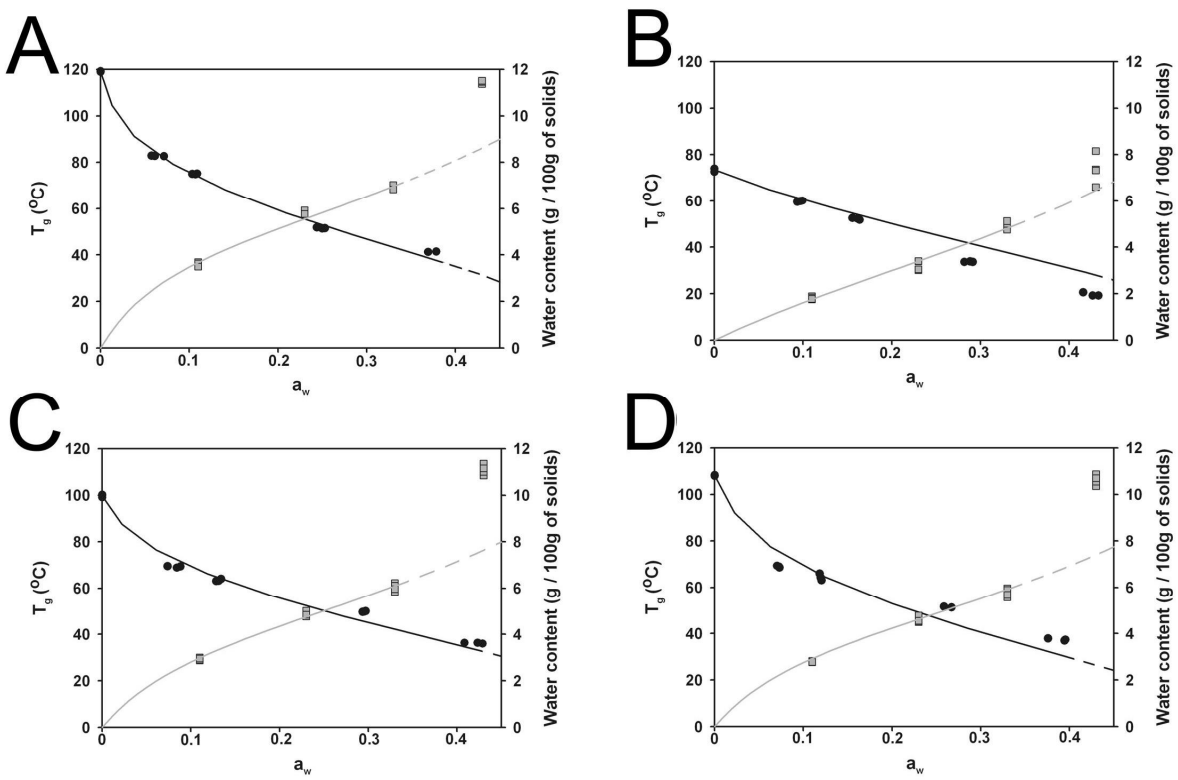


Figure 17. *The measured T_g (black circles, n = 3) and water content (gray squares, n = 4) values of trehalose (A), sucrose (B), melibiose (C) and cellobiose (D), as well as their GT (black line) and BET (gray line) fits. The GT and BET fits are dashed above the a_w range where the measurement values were no longer used in model fitting. Copyright © (2012) Springer Ltd., reprinted with permission.*

Trehalose, melibiose and cellobiose displayed classic type II sorption isotherms in the BET plots with relatively high C-values (Table 6), which are characteristic for amorphous compounds. On the other hand, less intense water sorption was observed in the isotherm of sucrose at low a_w, and as a result, its C-value was considerably lower. The m_0-values of all disaccharides were quite comparable, which could be interpreted to mean that the quantity of available water binding sites in the lyophilizates were close to equal. The measured T_gs of cellobiose and sucrose deviated more from the GT model than those of trehalose and melibiose, which affected their K-values. The reason for these poor fits were unknown, but a similar observation for sucrose has been made in another study as well (Hancock and Zografi, 1994). In that case, the authors concluded that the nature of the interactions between sucrose and water affect the free volume of the mixture differently than with other disaccharides, resulting in a lower T_g value than what is predicted by the GT model. There were some differences between the measured K-values and those found in literature, but this have been found to be common in case different measurement parameters have been used (Frank, 2007). The C-values are rarely reported in literature, and the measured value for trehalose differed significantly from what has been reported elsewhere (Zhang and Zografi, 2000), which may have been caused by different calculation methods.

Table 6. *The GT and BET model parameters measured (meas.) or acquired from literature (lit.) for the disaccharides studied, as well as the critical water contents [C_{crit} (H_2O), %-w/w], where T_g = 25°C. GT models were used to predict the C_{crit} (H2O) values for other disaccharides except for sucrose, where a 2nd order polynomial fit of the measured T_g values was used instead.*

	T_g (dry)		K		m_0		C		C_{crit} (H_2O)
disaccharide	meas.	lit.	meas.	lit.	meas.	lit.	meas.	lit.	meas.
cellobiose	108°C	77°C (1)	7.1	-	4.8	-	9.5	-	6.9 %
melibiose	100°C	95°C (2)	5.4	6.1 (4)	5.0	-	9.3	-	8.0 %
sucrose	74°C	70°C (2)	4.2	5.1 – 5.2 (5)	5.0	7.5 (6)	3.7	-	5.4 %
trehalose	119°C	121°C (3)	6.0	5.4 – 5.8 (5)	5.4	6.4 (7)	12.2	5.0 (7)	8.9 %

References: (1): Costantino et al. (1998), (2): Orford et al. (1990), (3): Hinrichs et al. (2005), (4): Roos (1997), (5): Frank (2007), (6): Wang et al. (2009), (7): Zhang and Zografi (2000).

The structural relaxation times of two of the most physically stable disaccharides (i.e. trehalose and melibiose) were studied as a function of water content by using ssNMR. In Figure 18 an increase in water content is denoted by an increase in T / T_g, and the results indicated that the T_1H of melibiose was higher than that of trehalose at a given T_g. As molecular mobility decreases with increasing relaxation rates, longer relaxation time was an indication of more restricted hydrogen mobility.

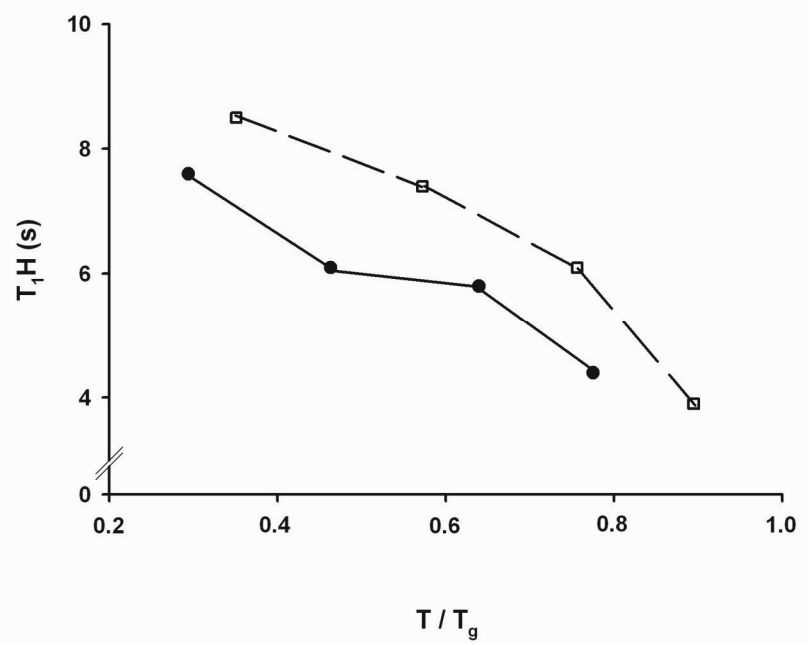


Figure 18. *The change in T_1H of trehalose (black circles) and melibiose (white squares) as a function of T_g. Copyright © (2012) Springer Ltd., reprinted with permission.*

5.3.4 Consequences of physical stability differences between excipients

The results of these experiments offered some possible explanations for the differences in β-galactosidase storage stabilities in different formulations. Cellobiose and sucrose were shown to inhibit protein activity loss less effectively than melibiose and trehalose at low

excipient concentrations (Fig. 12), and a similar division was observed regarding the crystallization tendencies of the disaccharides (Fig. 16). Using 730:1 excipient/protein molar ratio in the β-galactosidase studies resulted in lower lyophilizate mass (< 2 mg) than with 7300:1 (> 10 mg) or 15000:1 (> 20 mg) molar ratios. The initial average water content of the 730:1 molar ratio lyophilizates was also higher (≈ 2.7 %-w/w) than that of 7300:1 (≈ 1.6 %-w/w) or 15000:1 (≈ 1.3 %-w/w) molar ratio lyophilizates, and the water contents may have increased during storage. Higher water content could have led to a higher excipient crystallization rate in the samples with 730:1 excipient/protein molar, especially in the case of the relatively easily crystallizing compounds cellobiose and sucrose. However, small crystalline regions might have gone unnoticed in the XRPD analyses in such low mass lyophilizates (m < 2 mg). The underlying causes for the pronounced crystallization tendencies of cellobiose and sucrose were not certain, but in the case of sucrose its relatively low T_g (≈ 74°C) meant that its $C_{crit}(H_2O)$ was clearly lower than with the other disaccharides. Crystallization usually proceeds more quickly at $T_g < T < T_m$, and a lower water content was therefore necessary to depress its T_g below storage temperature. On the other hand, the water solubility of cellobiose has been shown to be relatively low compared to other sugars (Gray et al., 2003), and compounds with low aqueous solubility have sometimes been observed to possess a high crystallization tendency from the amorphous state (Roos, 1993).

The lower hydrogen mobility of melibiose could be one of the reasons why it was shown to crystallize later than trehalose when stored at RH 43%. Furthermore, the higher T_1H of melibiose might also have been an indication of its ability to form a glass matrix which restricted the mobility of β-galactosidase more effectively than trehalose. However, these were only some of the possible reasons for the differences between the cryo-/lyoprotecting efficacies of the two compounds. It was therefore necessary to study the protein-protecting efficacy of melibiose using another model protein, and to estimate whether the freeze drying process itself affects protein stability.

5.4 Studying the effect of freeze drying parameters and formulation on IgG stability (III)

Before carrying out further storage stability experiments, it was necessary to find out whether the freeze drying process had an effect on protein stability. Even though there is considerable evidence to suggest that exceeding T_g' or T_g during drying may not negatively affect the stability of some IgGs (Sarciaux et al., 1999; Schersch et al., 2010; Schersch et al., 2012), protein degradation might still occur if the formulation is not optimal (Izutsu et al., 1994). Therefore, potential hazards for polyclonal IgG stability during freeze drying were assessed by altering the processing conditions, namely primary drying pressure (Pprim) and secondary drying heating rate (ΔTsec), as well as the formulation composition.

5.4.1 Varying the processing conditions

Figure 19 shows the effect of Pprim and ΔTsec on product temperature during drying. Lowering the chamber pressure had a clear decreasing effect on sample temperature during primary drying due to an increase in sublimation rates. Heating the shelves at either 30°C/h or 60°C/h during secondary drying caused the samples to heat at relatively comparable rates, whereas using ΔTsec of 5°C/h resulted in relatively low sample heating rate. When using higher Pprim, collapses were observed in some samples (Fig. 20), and the average residual

lyophilizate water content was lower when dried at Pprim = 20 mTorr (2.6 ± 0.2%-w/w) than at Pprim = 100 mTorr (3.3 ± 0.5%-w/w). The PLS model formed from the water content results confirmed that Pprim had a statistically significant effect on residual lyophilizate water contents ($p < 0.05$). This suggested that ice sublimation was not always completed at the end of primary drying when using higher Pprim, because otherwise all samples should have contained similar amount of water at the end of primary drying. This may have caused the collapses to occur in some samples (Fig. 20), as the shelf temperature was increased during secondary drying while some samples still contained ice crystals.

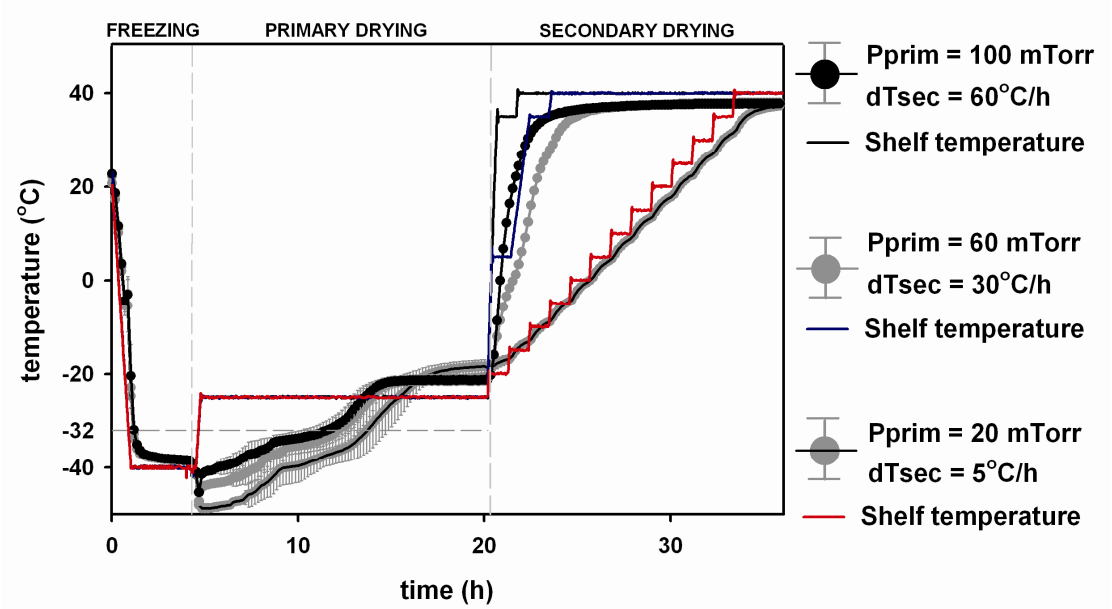


Figure 19. *The effect of Pprim and ΔTsec on average sample temperature (± SD), when measured with thermocouples. The T_g' of the formulation (-32°C) is marked with a dashed gray horizontal line, and shelf temperatures are shown as straight black, blue and red lines.*

The effects of freeze drying parameters on polyclonal IgG stability were analyzed with *in vitro* binding activity analysis by using ELISA, by measuring MRE changes at 217 nm wavelength with CD, by measuring Z-ave and PDI with DLS, and by measuring changes in soluble IgG monomer content with AF4. In order to check whether there were statistically significant changes in the responses due to freeze drying parameters, PLS models were created from the measurement results. The Q^2 and R^2 values for each response are shown in Table 7. Q^2 is generally considered a better indicator for model validity than R^2, because the goodness of fit may be increased simply by making the model more complicated, whereas this does not automatically result in better goodness of prediction. The Q^2 values of Z-ave and PDI indicate that the prediction accuracies of these models were relatively high, as does Figure 21, where the measured average Z-ave and PDI values are shown along with the calculated models. The figure shows that drying the IgG samples with lower Pprim resulted in significantly higher Z-ave and PDI ($p < 0.01$), which may have been caused by increased protein aggregation. The effect of ΔTsec on these responses was not statistically significant. The lack of fit regarding the other responses may have indicated that either Pprim and ΔTsec did not have any measurable effect on MRE at 217 nm wavelength, IgG monomer content or its *in vitro* binding activity, or then the measurement methods were not accurate enough.

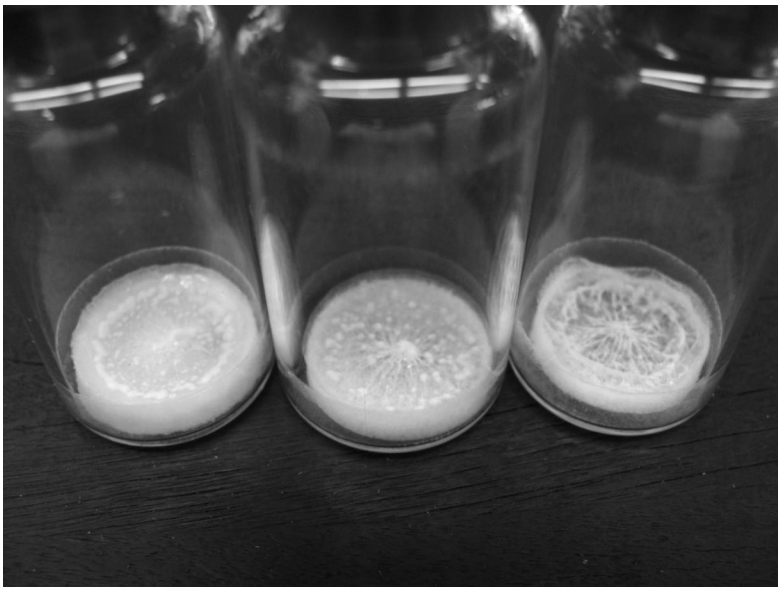


Figure 20. *Lyophilizates dried with a Pprim of 20 mTorr (left), 60 mTorr (center) and 100 mTorr (right).*

Table 7. *Evaluation of PLS model responses (analysis techniques shown in brackets) based on their goodness of fit (R^2) and goodness of prediction (Q^2) values.*

	%-act. (ELISA)	MRE 217 nm (CD)	Z-ave (DLS)	PDI (DLS)	Monomer-% (AF4)
R^2	0.06	0.37	0.64	0.81	0.23
Q^2	0.00	0.06	0.60	0.75	0.00

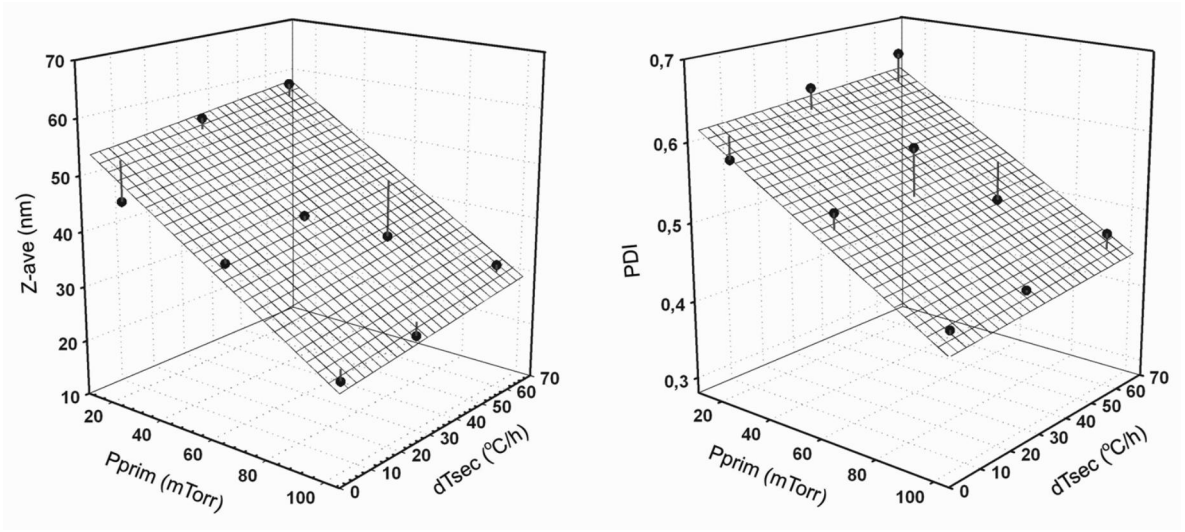


Figure 21. *The effect of Pprim and ΔTsec on Z-ave (left) and PDI (right) of rehydrated lyophilizates, when measured with DLS. The grids show the PLS-predicted effect of formulation parameters and the black dots the measured responses, while the gray lines depict the model deviation from measured values.*

5.4.2 Varying the formulation composition

In order to identify possible causes for the effect of Pprim on IgG aggregation, the formulation composition was varied. Figure 22 depicts the effect of drying different formulations at either Pprim = 20 mTorr or 100 mTorr. Including NaCl in the formulation caused a clear decrease in both Z-ave and PDI compared to using only trehalose and BSA as protective excipients. Furthermore, using Na-phosphate buffer was shown to increase Z-ave and PDI when drying at Pprim = 100 mTorr. Since sample temperature during drying was shown to decrease as a function of Pprim (Fig. 19), it is possible that physical state of Na-phosphate buffer was affected by the sample temperature. Even Na-phosphate buffers as dilute as 1 mM have been shown to crystallize as $Na_2HPO_4 \cdot 12H_2O$ when frozen to -50°C (Varshney et al., 2006). On the other hand, another component of the buffer, NaH_2PO_4, may remain amorphous during freezing in formulations containing several solutes, in which case it has a T_g' of -45°C (Chang and Randall, 1992). It is therefore possible that when the formulations containing Na-phosphate buffer were first frozen at -40°C, the freeze concentration of NaH_2PO_4 was not complete if the buffer was excluded as its own amorphous phase. Drying at Pprim = 20 mTorr cooled the samples temporarily to approximately -48°C (Fig. 19), which may then have resulted in additional freeze concentration and buffer crystallization compared to drying at higher pressure. This may have resulted in more pronounced change in the physical state of the Na-phosphate buffer (i.e. from amorphous to crystalline), and subsequently altered protein conformation. However, the verification of buffer crystallization from the lyophilizates was not successful, because crystalline as $Na_2HPO_4 \cdot 12H_2O$ tends to become amorphous when it is dehydrated during primary drying (Pyne et al., 2003). Another possible reason for the effect of formulation parameters on Z-ave and PDI is that the excipients affected the amount of air bubbles that were formed during rehydration, but this should not have accounted for the difference observed as a function Pprim (Figs. 21 and 22).

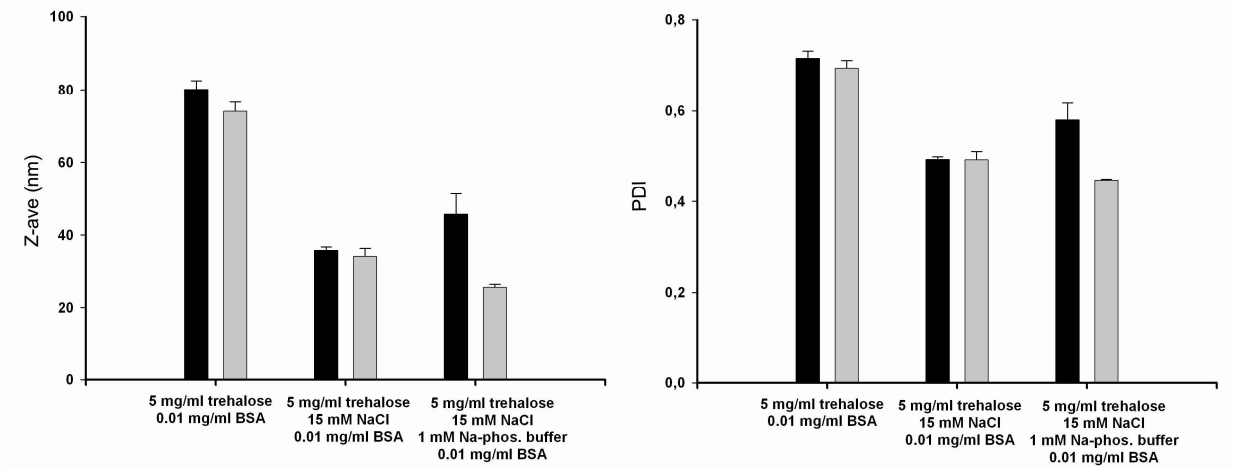


Figure 22. *The effect of formulation and processing parameters on Z-ave (left) and PDI (right). The black bars represent freeze drying with Pprim = 20 mTorr and the gray bars with Pprim = 100 mTorr, with ΔTsec being 5C°/h in both cases.*

5.4.3 Factors to be taken into account when freeze drying IgG-class proteins

Product collapse did not seem to result in decreased polyclonal bovine IgG stability immediately after freeze drying. Furthermore, longer holding time at 40°C during secondary

drying (i.e. using higher ΔTsec) did not cause considerable monomer loss, aggregation or secondary structure alterations. Freeze drying parameters only appeared to have an effect on polyclonal IgG stability when a crystallizing excipient (Na-phosphate buffer) was used in the formulation. Therefore non-crystallizing buffers should be preferred. Here increasing the lyophilizate residual water content (at 2.5 – 3.3%-w/w interval) did not lead to increased protein degradation, but this does not mean that changes in protein structure could not have occurred with lower or higher water contents than this. Including NaCl in the formulation reduced average particle size and polydispersity in the rehydrated solutions. This could have been caused by its effect on the protein surface potential, but perhaps a more likely cause was that a smaller number of air bubbles were formed during lyophilizate rehydration when NaCl was used in the formulation.

Changes in the Z-ave and PDI of rehydrated samples seemed to be more clear indicators of IgG instability compared to secondary structure alterations, monomer loss or *in vitro* binding activity. This was to be expected, since aggregation tends to be a common response to many different types of stresses in IgG-class proteins (Hawe et al., 2009; Thirumangalathu et al., 2009; Tyagi et al., 2009). It is also recommended to use several orthogonal analysis techniques in protein stability studies to properly characterize the properties of the aggregates that have been formed (Carpenter et al., 2010).

5.5 Comparing the efficacies of trehalose and melibiose as cryo-/lyo-protecting excipients for rituximab during freeze drying and storage (IV)

Further comparison between the protein-protecting efficacies of trehalose and melibiose was carried out by freeze drying rituximab, a chimeric mouse/human IgG1κ mAb, while using either of the two disaccharides as cryo-/lyoprotecting excipients. Rituximab used in the study is marketed with the trade name MabThera® (F. Hoffmann-La Roche Ltd., Basel, Switzerland), which is used in the treatment of Non-Hodgkin lymphoma, chronic lymphocytic leukemia and severe rheumatoid arthritis. One topic of interest in the experiments was to find out whether the water content of the lyophilizates affects rituximab stability during storage, and to take this into consideration, the lyophilizates were stored in loosely stoppered vials at different relative humidity atmospheres.

5.5.1 Protein purification and lyophilizate physical properties

In addition to the API, MabThera® also contains 7.35 mg/ml sodium citrate, 9 mg/ml sodium chloride and 0.7 mg/ml PS80 as excipients, and the removal of the original formulation excipients was carried out with size-exclusion fractionation columns. However, the PS80 quantitation measurements after fractionation showed that the process did not completely purify the surfactant from the solution, removing only approximately 37% of the total PS80 content. Therefore, the solutions contained 1 mg/ml rituximab, 50 mg/ml disaccharide (either trehalose or melibiose), 10 mM Na-citrate buffer (pH 6.3) and 0.033 mg/ml Ps80 before freeze drying.

The lyophilizate water contents and T_gs before and after storage at different relative humidity atmospheres are shown in Table 8. Storage at RH 5% did not lead to clear changes in water content or T_g, but increased water content at RH 11% and 23% resulted in greated T_g depression due to water plasticization. T_g was not exceeded in either formulation during storage, and no lyophilizate collapses were observed. Similarly, excipient crystallization was

not detected with XRPD in the lyophilizates during storage at any relative humidity atmosphere when analyzed monthly (data not shown).

Table 8. *Water contents and glass transition temperatures (T_g) of lyophilized trehalose and melibiose formulations before and after the 90-day storage under different relative humidity atmospheres. The water content measurement standard deviations before storage for trehalose and melibiose formulations were ±0.3% and ±0.1%, respectively.*

	Water content (w/w)				T_g			
Excipient used	Before storage	After storage, RH 5%	After storage, RH 11%	After storage, RH 23%	Before storage	After storage, RH 5%	After storage, RH 11%	After storage, RH 23%
trehalose	2.3%	2.5%	4.1%	5.9%	91°C	90°C	74°C	56°C
melibiose	2.1%	2.3%	3.7%	5.9%	82°C	86°C	67°C	57°C

5.5.2 Effects of freeze drying and storage on rituximab structure and sample particle contents

Freeze drying caused clear alterations in rituximab CD spectra, especially in the 190 – 200 nm wavelength region (Fig. 23). According to literature, these spectral changes may be attributed to an increase in the protein α-helix content (MRE intensification around 190 – 200 nm), as well as to a decrease in anti-parallel β-sheet content (MRE intensification at 218 nm) (Kelly et al., 2005). The changes in the 190 – 200 nm wavelength region were more pronounced in the trehalose formulation. Freeze drying and rehydration also increased the amount of < 2 µm diameter particles in the LO analyses (Fig. 24, A) in both formulations, but the increase in particle concentration was more significant in the trehalose formulation. Similarly, drying and rehydration increased the amount of < 1 µm diameter particles in the NTA analyses from $0.1*10^9$ (± $0.02*10^9$) particles per container before freeze drying to $1.0*10^9$ (± $0.5*10^9$) and $0.4*10^9$ (± $0.2*10^9$) particles per container in the trehalose and melibiose formulations, respectively. When analyzed with DLS, the rituximab solution before freeze drying had Z-ave of 12 ± 0.7 nm and PDI of 0.3 (± 0.0). Drying and rehydration increased Z-ave and PDI in both trehalose (Z-ave 82 ± 16 nm, PDI 1.0 ± 0.0) and melibiose formulations (Z-ave = 14 ± 0.2 nm, PDI = 0.4 ± 0.0), but the effect was much less intensive in the latter. The particle analysis methods (LO, NTA and DLS) cannot be used to analyze particle composition, so it is not known whether they consisted of aggregated protein, silicon oil or air. Therefore, the particle counts and sizes measured with these techniques may not represent the actual protein aggregate contents of the rehydrated solutions. It should also be noted that even though LO is sometimes used to quantify > 1 µm particles (Hawe et al., 2009), it has been stated that the method might be inaccurate for the analysis of particles < 2 µm (Singh et al., 2010).

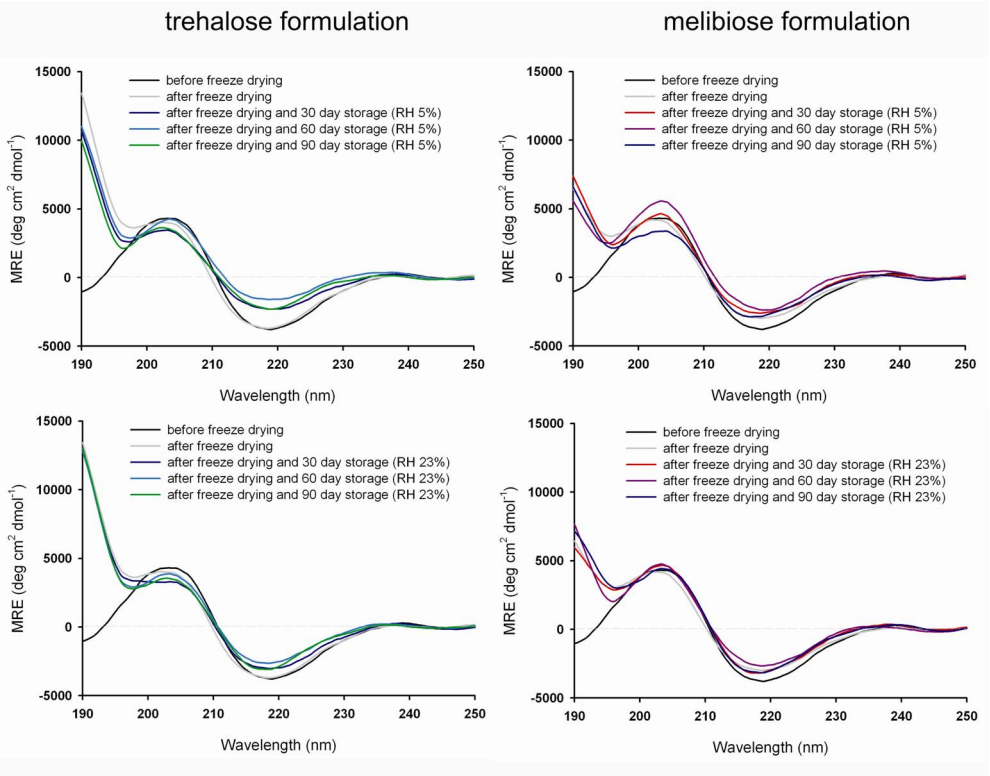


Figure 23. *CD spectra of rituximab before and after freeze drying in trehalose (left) and melibiose (right) formulations, and after storage at RH 5% (upper) or RH 23% (lower) for up to 90 days. Copyright © (2013) Wiley-Blackwell Ltd., reprinted with permission.*

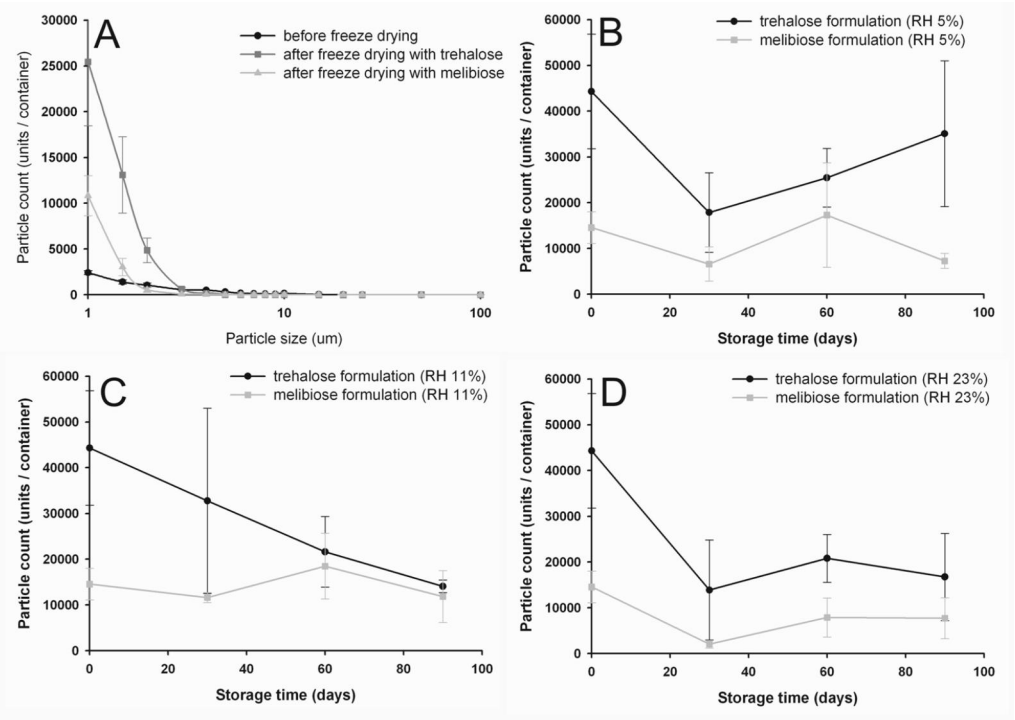


Figure 24. *> 1 µm particle counts (measured with LO) in trehalose and melibiose formulations. A: The effect of freeze drying on particles counts and size distributions (logarithmic particle size scale). B-D: The effect of storage at different relative humidity atmospheres on particles counts. Copyright © (2013) Wiley-Blackwell Ltd., reprinted with permission.*

HP-SEC analyses (Fig. 25) indicated that a small amount of rituximab fragments with $M_w \approx 80$ and 30 kDa (elution times approximately 25 and 26 min) were present in the formulations after freeze drying. Based on their molar masses, these fragments may have consisted of one heavy and one light IgG chain ($M_w \approx 80$ kDa) and a single light chain ($M_w \approx 30$ kDa). Based on the fluorescence detector results, the concentration of rituximab fragments with $M_w \approx 30$ kDa was 0.9% ± 0.02% in the trehalose and 0.03% ± 0.002% in the melibiose formulation of the total recovered rituximab content after freeze drying. This suggested that freeze drying in the presence of trehalose caused more rituximab fragmentation. SDS-PAGE analyses confirmed the presence of these fragments before and after freeze drying in both formulations (data not shown), but their concentrations could not be compared due to the semi-quantitative nature of the method.

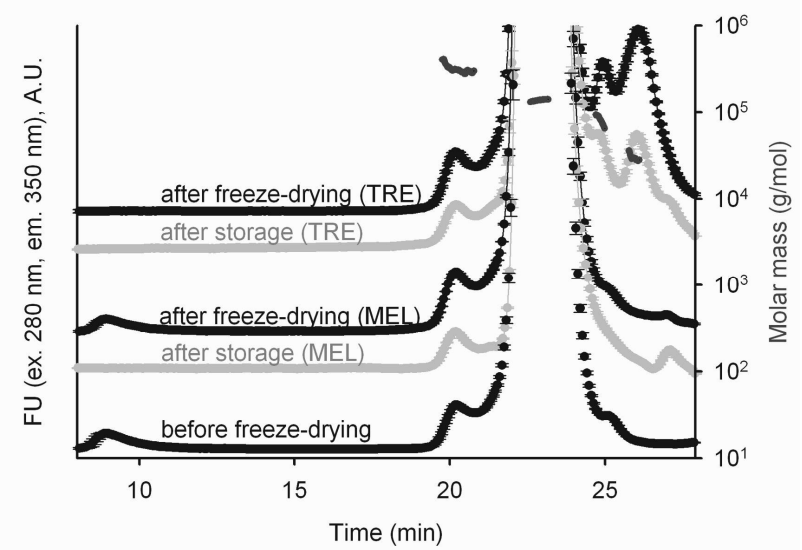


Figure 25. *HP-SEC fluorescence detector signals of rituximab before and after freeze drying in trehalose (TRE) and melibiose (MEL) formulations, and after 90-day storage at RH 23%. The molar mass values have been calculated from UV and MALLS signals. Copyright © (2013) Wiley-Blackwell Ltd., reprinted with permission.*

During storage, the concentration of > 1 µm diameter particles remained generally higher in the trehalose than in the melibiose formulation (Fig. 24, B-D), even though the particle counts appeared to decrease in the trehalose formulation during storage at RH 11% and 23%. The < 1 µm particle counts increased clearly in the trehalose formulation when stored at RH 5% and 11%, whereas only a small increase was observed in the melibiose formulation at RH 5% (Fig. 26). It is noteworthy that in the case of specifically particle-rich samples (e.g. in trehalose formulation samples that were stored at RH 5% for 30 days or longer), the solutions analyzed with NTA had to be diluted before measurement. This may have contributed to measurement error, because the sample dilution may break down protein aggregates or create new ones, or lead to air bubble formation. Still, the secondary structure changes were aggravated more in both formulations when stored at RH 5% than when stored at RH 23% (Fig. 23). The HP-SEC and SDS-PAGE analyses did not show significant changes occurring in either formulation, but the recovered 30 kDa fragment content appeared to decrease from 0.9% to 0.6% (± 0.02%) in the trehalose formulation during storage at RH 23%. No changes in SDS-PAGE results were observed during storage (data not shown).

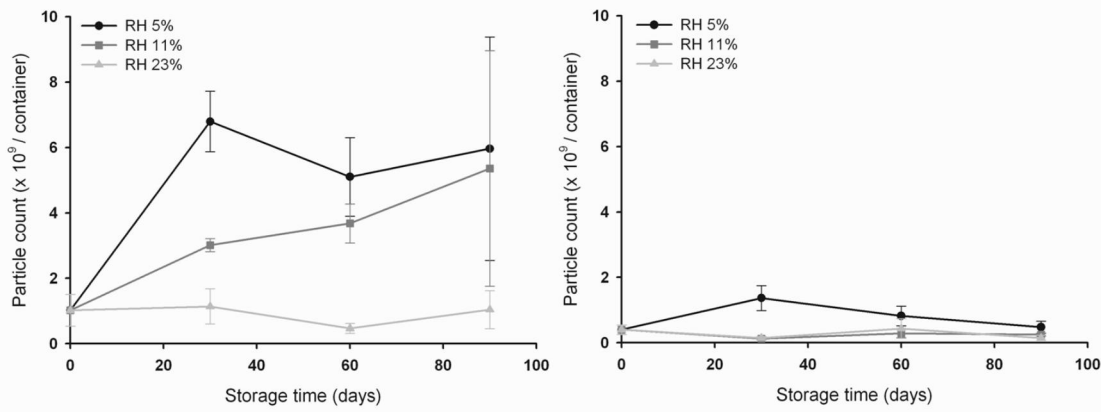


Figure 26. *< 1 µm particle counts (measured with NTA) in trehalose (left) and melibiose (right) formulations during storage at different relative humidity atmospheres. Copyright © (2013) Wiley-Blackwell Ltd., reprinted with permission.*

5.5.3 Factors affecting rituximab stability during and after drying

It was found that there were clear differences in rituximab stability during freeze drying depending on whether trehalose or melibiose was used as the cryo-/lyoprotecting excipient. The more significant rituximab aggregation, fragmentation and secondary structure alteration behavior observed in the trehalose formulation was not caused by differences in lyophilizate water contents between the two formulations. However, the storage stability studies at RH 5%, especially the CD and NTA results, indicated that rituximab storage stability was poor with relatively low lyophilizate water contents (≈ 2-3 %-w/w) compared to when the lyophilizate water content was higher. This coupled with the intense secondary structure alterations observed in both formulations as a result of freeze drying might mean that the protein was susceptible to drying-induced instability. Similar results regarding freeze-dried mAb stability in low residual water content lyophilizates have sometimes been observed. For example, a certain IgG_1 class protein was shown to exhibit decreased storage stability in lyophilizates with water content below 3% (Chang et al., 2005). On the other hand, the storage stability of another mAb seemed to be relatively unaffected by the lyophilizate water content at 1 - 8.4% interval (Cleland et al., 2001). Therefore it is likely that the effect of low water content on mAb storage stability is dependent on the protein in question.

The analytical techniques used here could not show that storage at higher relative humidity atmospheres would have resulted in increased rituximab degradation. If anything, the storage stability of the protein seemed to be better at RH 23% than at RH 5% in both formulations. However, the analytical methodologies used here might not have been accurate enough to detect small concentrations of Maillard reaction byproducts, and analysis methods capable of detecting changes in protein glycation, such as those developed by Fisher et al. (2008), should be additionally employed in future studies. It is also likely that Maillard reaction would become a more important degradation route for rituximab at a higher temperature or humidity than what was used in these studies. Still, the st0rage conditions used here can still be considered more stressed than what marketed lyophilizates are normally subjected to before administration to patients. This is because commercial freeze-dried products would rarely contain such water contents as seen here (up to 6%-w/w), as the primary package protects the product from moisture uptake. Here the vials were stored loosely stoppered so that water sorption could occur. The storage conditions were chosen so

that they would not induce lyophilizate collapse due to the depression of T_g below the storage temperature, because reaction kinetics at $T > T_g$ might not represent those at $T < T_g$. While the ICH guideline Q5C on the stability testing of biotechnological products suggests that accelerated storage stability studies are important for pinpointing potential hazards for the stability of protein drug products, it also states that their actual expiration times should be defined based on real-time data at intended storage conditions (ICH, 1995). Therefore, Maillard reaction kinetics studies conducted at elevated temperatures might not correspond with the actual shelf life of a rituximab drug product containing melibiose.

5.6 Using melibiose as a freeze drying excipient (I, II, IV)

Prior to these experiments, there were no published studies available where melibiose (6-O-α-D-galactopyranosyl-D-glucopyranose) would have been used to protect proteins from degradation during freeze drying and the subsequent storage. Also, there was very little information on its stability in amorphous state. Here, the cryo-/lyoprotective efficacy of melibiose was shown to be comparable to trehalose when using β-galactosidase as the model protein (I), or better when using rituximab (IV).

One reason for this may have been the physical stability of amorphous melibiose. Its crystallization rate in room temperature with water contents up to ≈ 10%-w/w was shown to be relatively slow compared to cellobiose, sucrose and trehalose (II). The molecular mobility of amorphous melibiose (determined based on T_1H) was also slower than that of trehalose, when the T_gs of the disaccharide/water mixtures were comparable. This means that the amorphous matrix which melibiose forms around proteins during the freezing step might be relatively viscous and stable, which could effectively inhibit protein unfolding and reactivity.

On the other hand, the studies conducted with rituximab (IV) suggested that melibiose may have been more effective than trehalose in protecting rituximab from degradation under dry conditions (RH 5%). Therefore its efficacy in partially substituting water as a hydrogen bond former with hydrophilic protein surface moieties may have been better than that of trehalose. The hydrogen bond formation tendencies of different disaccharides can be assessed to some degree based on their molecular structures. As shown in Figure 13 (chapter 5.3.1), the molecular structure of melibiose differs from that of trehalose in two distinct ways. While trehalose is formed of two glucose units (linked together by an α-1,1 glycosidic bond), melibiose consists of one glucose and one galactose unit (connected by an α-1,6 glycosidic bond). Secondly, the glycosidic bond in melibiose connects to the exocyclic methyl alcohol group of the glucose subunit, creating a two-carbon atom bridge between the monosaccharide rings, whereas this bridge consists of only one carbon atom in many of the other disaccharides studied here. Interestingly, isomaltulose (6-0-α-D-glucopyranosyl-D-fructose) consists of glucose and fructose subunits linked together by a similar α-1,6 glycosidic bond as in melibiose. As can be seen from Figure 12 (chapter 5.2.1), isomaltulose was also effective in protecting β-galactosidase from activity loss, despite being a reducing disaccharide with a relatively low T_g (Table 2, chapter 2.5.1). Whether such a two-carbon atom bridge between the monosaccharide subunits results in improved protein protective efficacy can only be speculated at this point. However, it might improve the flexibility of the disaccharide backbone, allowing an increased number of hydroxyl groups in a single molecule to form hydrogen bonds with protein surface moieties compared to a more rigid molecule. Additional studies are necessary to evaluate whether such structural properties can affect the cryo-/lyoprotective efficacies of disaccharides.

Overall, these studies represent very early stage attempts of using excipients in freeze-dried formulations which have not been approved by the regulatory authorities. Therefore, the feasibility of using melibiose in pharmaceutical protein drug products cannot be assessed based on these results alone. The next logical step towards proving or disproving that it could be used would be to analyze whether differences in protein glycation are observed when melibiose is used as opposed to a non-reducing disaccharide. Furthermore, despite melibiose being abundantly present in many commonly ingested food products such as those containing soy beans (Caplice and Fitzgerald, 1999), conclusive toxicity studies would be needed to determine whether the compound is safe to be used as a pharmaceutical excipient.

6 Conclusions

In this thesis several disaccharides and polyalcohols were studied as protein-protecting excipients in freeze-dried formulations. Based on the enzymatic activity retention studies carried out using β-galactosidase as the model protein, disaccharides were generally better than polyalcohols in inhibiting activity loss during storage. One reason for this may have been the relatively low T_gs and high crystallization tendencies of many polyalcohols. Also, in the case of disaccharides, storage at high relative humidity atmospheres brought out differences in excipient crystallization tendencies. Despite their relatively good efficacies in protecting β-galactosidase from activity loss during storage, sucrose and cellobiose were shown to crystallize more easily than trehalose and melibiose. While such a tendency would probably not manifest in freeze-dried protein formulations under normal storage conditions, storage at high temperatures or water release from vial stoppers might result in the crystallization of unstable excipients.

The efficacy of melibiose as a protein-protecting excipient was not attributable only to its low crystallization tendency, however. Compared to trehalose, melibiose was superior in inhibiting rituximab degradation during freeze drying and storage in 25°C in relative humidity atmospheres up to 23%. Possible reasons for this include its relatively slow hydrogen mobility, as well as its hydrogen bond formation properties, which may allow it to effectively replace water on protein surfaces during dehydration. The freeze drying stability of rituximab was not thoroughly explored by using different formulations or freeze drying parameters, because the main objective was to compare trehalose and melibiose as protein-protecting excipients. Still, these studies gave an indication that rituximab might be susceptible to drying-induced degradation, even though additional studies would be needed to assess whether this might occur in other formulations as well.

Based on the freeze drying experiments carried out with polyclonal IgG, it could be hypothesized that the stability of the protein did not depend heavily on freeze drying parameters. However, this does not always hold true when using crystallizing excipients in the formulations. In the case of polyclonal IgG, different primary drying pressure or secondary drying heating rate did not affect protein stability unless Na-phosphate was used in the formulation. In that case, using a lower primary drying pressure led to an increase in protein aggregation, which was most likely caused by increased disodium hydrogen phosphate dodecahydrate crystallization. The combination of low sample temperature and Na-phosphate buffer should therefore be avoided. Lyophilizate collapse during freeze drying did not appear to affect protein stability negatively.

These results encourage further studies on “new” freeze drying excipients. Although many carbohydrates have been extensively studied for other applications and are readily available as pure substances, the published studies regarding the use of compounds other than the most common ones as freeze drying excipients are scarce. Broadening the horizons in this field may lead to new innovations in API stabilization and processing. Instead of declaring certain compounds such as reducing disaccharides or easily crystallizing buffers unfit as excipients in certain products, it would be important to evaluate whether their properties affect API stability under relevant processing and storage conditions. It would also be interesting to examine how often accelerated stability studies correlate with actual storage times in the case of protein pharmaceuticals. Nevertheless, regulatory guidelines regarding accelerated API stability measurements must be borne in mind, because they cannot be disregarded when applying for marketing authorization for a new pharmaceutical product.

References

Adam, G., Gibbs, J., 1965. On the temperature dependence of cooperative relaxation properties in glass-forming liquids. J. Chem. Phys. 43: 139-146.

Adamo, M., Dick L., Qiu, D., Lee, A.-H., Devincentis, J., Cheng, K.-C., 2010. A simple reversed phase high-performance liquid chromatography method for polysorbate 80 quantitation in monoclonal antibody drug products. J. Chromatogr. B 878: 1865-1870.

Akkati, S., Sam, K., Tungha, G., 2011. Emergence of Promising Therapies in Diabetes Mellitus. J. Clin. Pharmacol. 51(6): 796-804.

Alexander, A., Hughes, D., 1995. Monitoring of IgG antibody thermal stability by micellar electrokinetic capillary chromatography and matrix-assisted laser desorption/ionization mass spectrometry. Anal. Chem. 67: 3626-3632.

Allison, S., Dong, A., Carpenter, J., 1996. Counteracting effects of thiocyanate and sucrose on chymotrypsinogen secondary structure and aggregation during freezing, drying and rehydration. Biophys. J. 71(4): 2022-2032.

Arrondo, J., Muga, A., Castresana, J., Bernabeu, C., Goni. F., 1989. An infrared spectroscopic study of β-galactosidase structure in aqueous solutions. FEBS Letters 252(1-2): 118-120.

BCC-Research, 2012. Antibody Drugs: Technologies and Global Markets (accessed 14.11.2012). Available online at: http://www.bccresearch.com/report/antibody-drugs-technologies-markets-bio016h.html.

Bee, J., Stevenson, J., Mehta, B., Svitel, J., Pollastrini, J., Platz, R., Freund, E., Carpenter, J., Randolph, T., 2009. Response of a concentrated monoclonal antibody formulation to high shear. Biotechnol. Bioeng. 103(5): 936-943.

Bell, L., 2007. Moisture effects on food's chemical stability. In: Barbosa-Cánovas, G., Fontana, A., Schmidt, S., Labuza, T. (Ed.): Water activity in foods. Blackwell Publishing, Oxford, UK, pp. 173-198.

Bhatnagar, B., Bogner, R., Pikal, M., 2007. Protein stability during freezing: Separation of stresses and mechanisms of protein stabilization. Pharm. Dev. Technol. 12: 505-523.

Bhugra, C., Pikal. M., 2008. Role of thermodynamic, molecular, and kinetic factors in crystallization from the amorphous state. J. Pharm. Sci. 97(4): 1329-1349.

Branden, C., Tooze, J., 1999. Introduction to protein structure. Garland publishing, New York, USA.

Bruel, L., Sulzenbacher, G., Tison, M., Pujol, A., Nicoletti, C., Perrier, J., Gallnler, A., Ropartz, D., Fons, M., Pompeo, F., Giardina, T., 2011. α-galactosidase/sucrose kinase (AgaSK), a novel bifunctional enzyme from the human microbiome coupling galactosidase and kinase activities. J. Biol. Chem. 286(47): 40814-40823.

Brunauer, S., Emmett, P., Teller, E., 1938. Adsorption of gases in multimolecular layers. J. Am. Chem. Soc. 60: 309-319.

Butterworth, S., Lamzin, V., Wigley, D., Derrick, J., Wilson, K., 1998. Anisotropic refinement of a protein G domain at 1.1 ångstrom resolution. The Protein Databank in Europe (accessed 13.11.2012). Available online at: http://www.ebi.ac.uk/pdbe/.

Caplice, E., Fitzgerald, G., 1999. Food fermentations: Role of micro-organisms in food production and preservation. Int. J. Food Microbiol. 50: 131-149.

Carba, V., Vázquez-Contreras, E., Moreno, A., Arreguin-Espinosa, R., 2008. The effect of sulfhydryl groups and disulphide linkage in the thermal aggregation of Z19 α-zein. Biophys. Biochim. Acta 1784: 1028-1036.

Carpenter, J., Crowe, J., 1989. An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. Biochem. 28(9): 3916-3922.

Carpenter, J., Randolph, T., Jiskoot, W., Crommelin, D., Middaugh, C., Winter, G., Fan, Y-X., Kirshner, S., Verthelyi, D., Kozlowski, S., Clouse, K., Swann, P., Rosenberg, A., Cherney, B., 2009. Overlooking subvisible particles in therapeutic protein products: Gaps that may compromise product quality. J. Pharm. Sci. 98(4): 1201-1205.

Carpenter, J., Randolph, T., Jiskoot, W., Crommelin, D., Middaugh, C., Winter, G., 2010. Potential inaccurate quantitation and sizing of protein aggregates by size exclusion chromatography: Essential need to use orthogonal methods to assure the quality of therapeutic protein products. J. Pharm. Sci. 99(5): 2200-2208.

Chang, B., Fischer, N., 1995. Development of an efficient single-step freeze-drying cycle for protein formulations. Pharm. Res. 12(6): 831-837.

Chang, B., Randall, C., 1992. Use of subambient thermal analysis to optimize protein lyophilization. Cryobiology 29: 632-656.

Chang, L., Shepherd, D., Sun, J., Tang, X., Pikal, M., 2005. Effect of sorbitol and residual moisture on the stability of lyophilized antibodies: Implications for the mechanism of protein stabilization in the solid state. J. Pharm. Sci. 94(7): 1445-1455.

Chatterjee, K., Shalaev, E., Suryanarayanan, R., 2005. Partially crystalline systems in lyophilization: II. Withstanding collapse at high primary drying temperatures and impact on protein activity recovery. J. Pharm. Sci. 94(4): 809-820.

Cleland, J., Lam, X., Kendrick, B., Yang, J., Yang, T-H., Overcashier, D., Brooks, D., Hsu, C., Carpenter, J., 2001. A specific molar ratio of stabilizer to protein is required for storage stability of a lyophilized monoclonal antibody. J. Pharm. Sci. 90(3): 310-321.

Cooper, E., Knutson, K., 1995. Fourier transform infrared spectroscopy investigations of protein structure. In: Herron J., Jiskoot W., Crommelin D. (Ed.): Physical methods to characterize pharmaceutical proteins. Plenum Press, New York, USA, pp. 101-143.

Costantino, H., Carrasquillo, K., Cordero, R., Mumenthaler, M., Hsu, C., Griebenow, K., 1998. Effect of excipients on the stability and structure of lyophilized recombinant human growth hormone. J. Pharm. Sci. 87(11): 1412-1420.

Crowe, L., Reid, D., Crowe, J., 1996. Is trehalose special for preserving dry biomaterials? Biophys. J. 71(4): 2087-2093.

De Beer, T., Allesø, M., Goethals, F., Coppens, A., Vander Heyden, Y., Lopez De Diego, H., Rantanen, J., Verpoort, C., Vervaet, C., Remon, J., Baeyens, W., 2007. Implementation of a process analytical technology system in a freeze-drying process using raman spectroscopy for in-line process monitoring. Anal. Chem. 79(21): 7992-8003.

Dong, A., Huang, P., Caughey, W., 1990. Protein secondary structures in water from second-derivative amide I infrared spectra. Biochem. 29(13): 3303-3308.

Ediger, M., Angell, C., Nagel, S., 1996. Supercooled liquids and glasses. J. Phys. Chem. 100(31): 13200-13212.

Eppler, A., Weigandt, M., Hanefeld, A., Bunjes, H., 2010. Relevant shaking stress conditions for antibody preformulation development. Eur. J. Pharm. Biopharm. 74(2): 139-147.

Etter, M., 1990. Encoding and decoding hydrogen-bond patterns of organic compounds. Acc. Chem. Res. 23(4): 120-126.

Filipe, V., Hawe, A., Jiskoot, W., 2010a. Critical evaluation of nanoparticle tracking analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates. Pharm. Res. 27(5): 796-810.

Filipe, V., Hawe, A., Schellekens, H., Jiskoot, W., 2010b. Aggregation and immunogenicity of therapeutic proteins. In: Wang W., Roberts C. (Ed.): Aggregation of therapeutic proteins. John Wiley & Sons Inc., New Jersey, USA, pp. 403-433.

Filipe, V., Jiskoot, W., Basmeleh, A., Halim, A., Schellekens, H., Brinks, V., 2012. Immunogenicity of different stressed IgG monoclonal antibody formulations in immune tolerant transgenic mice. mAbs 4(6): 740-752.

Fisher, S., Hoernschemeyern J., Mahler, H-C., 2008. Glycation during storage and administration of monoclonal antibody formulations. Eur. J. Pharm. Biopharm. 70: 42-50.

Frank, G., 2007. Measurement analysis of glass transition temperature for sucrose and trehalose aqueous solutions. J. Phys. Chem. Ref. Data 36(4): 1279-1285.

Franks, F., 1998. Freeze-drying of bioproducts: putting principles into practice. Eur. J. Pharm. Biopharm. 45(3): 221-229.

Franks, F., 2007. Freeze-drying of pharmaceuticals and biopharmaceuticals. The Royal Society of Chemistry, Cambridge, UK.

Gamble, C., 1966. The role of soluble aggregates in the primary immune response of mice to human gamma globulin. Int. Arch. Allergy Appl. Immunol. 30(5): 446-455.

Gomez, G., Pikal, M., Rodriguez-Hornedo, N., 2001. Effect of initial buffer composition on pH changes during far-from-equilibrium freezing of sodium phosphate buffer solutions. Pharm. Res. 18(1): 90-97.

Gordon , M., Taylor, J., 1952. Ideal copolymers and the second-order transitions of synthetic rubbers. I. Non-crystalline copolymers. J. Appl. Chem. 2: 493-500.

Goto, Y., Fink, A., 1989. Conformational states of β-lactamase: Molten-globule states at acidic and alkaline pH with high salt. Biochem. 28: 945-952.

Gray, M., Converse, A., Wyman, C., 2003. Sugar monomer and oligomer solubility: data and predictions for application to biomass hydrolysis. Appl. Biochem. Biotech. 105(1-3): 179-193.

Hagen, S., Hofrichter, J., Eaton, W., 1995. Protein reaction kinetics in a room-temperature glass. Science 269(5226): 959-962.

Hancock , B., Zografi, G., 1994. The relationship between the glass transition temperature and the water content of amorphous pharmaceutical solids. Pharm. Res. 11(4): 471-477.

Haque, M., Roos, Y., 2003. Water plasticization and crystallization of lactose in spray-dried lactose/protein mixtures. J. Food Sci. 69(1): 23-29.

Hatley, R., Franks, F., Mathias, S., 1987. The stabilization of labile biochemicals by undercooling. Process Biochem. 22(6): 169-172.

Hawe, A., Kasper, J., Friess, W., Jiskoot, W., 2009. Structural properties of monoclonal antibody aggregates induced by freeze–thawing and thermal stress. Eur. J. Pharm. Biopharm. 38: 79-87.

Hawe, A., Filipe, V., Jiskoot, W., 2010. Fluorescent molecular rotors as dyes to characterize polysorbate-containing IgG formulations. Pharm. Res. 27(2): 314-326.

Hinrichs, W., Sanders, N., De Smedt, S., Demeester, J., Frijlink, H., 2005. Inulin is a promising cryo- and lyoprotectant for PEGylated lipoplexes. J. Control. Release 103(2): 465-479.

Hodge, I. 1987. Effects of annealing and prior history on enthalpy relaxation in glassy polymers. 6. Adam—Gibbs formulation of nonlinearity. Macromolecules 20: 2897-2908.

ICH, 1995. Quality of biotechnological products: Stability testing of biotechnological/biological products (Q5C). The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (accessed 14.11.2012). Available online at: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5C/Step4/Q5C_Guideline.pdf.

ICH. 1999. Test procedures and acceptance criteria for biotechnological/biological products (Q6B). The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (accessed 14.11.2012). Available online at:http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q6B/Step4/Q6B_Guideline.pdf

ICH. 2003. Stability testing of new drug substances and products, revision 2 (Q1A-R2). The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (accessed 7.1.2013). Available online at: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q1A_R2/Step4/Q1A_R2__Guideline.pdf

Ito, H-O., Nakashima, T., So, T., Hirata, M., Inoue, M., 2003. Immunodominance of conformation-dependent B-cell epitopes of protein antigens. Biochem. Biophys. Res. Commun. 308: 770-776.

Izutsu, K., Yoshioka, S., Terao, T., 1993. Decreased protein-stabilizing effects of cryoprotectants due to crystallization. Pharm. Res. 10(8): 1232-1237.

Izutsu, K., Yoshioka, S., Terao, T., 1994. Effect of mannitol crystallinity on the stabilization of enzymes during freeze-drying. Chem. Pharm. Bull. 42(1): 5-8.

Jaenicke, R., 1990. Protein structure and function at low temperatures. Phil. Trans. R. Soc. Lond. B 326: 535-553.

Jaenicke, R., 1991. Protein stability and molecular adaptation to extreme conditions. Eur. J. Biochem. 202: 715-728.

Jahn, E-M., Schneider, C., 2009. How to systematically evaluate immunogenicity of therapeutic proteins – Regulatory considerations. New Biotech. 25(5): 280-286.

Kasper, J., Friess, W., 2011. The freezing step in lyophilization: Physico-chemical fundamentals, freezing methods and consequences on process performance and quality attributes of biopharmaceuticals. Eur. J. Pharm. Biopharm. 78(2): 248-263.

Kauzmann, W. 1948. The nature of the glassy state and the behavior of liquids at low temperatures. Chem. Rev. 43: 219-256.

Kelly, S., Jess, T., Price, N., 2005. How to study proteins by circular dichroism. Biochim. Biophys. Acta 1751: 119-139.

Kerwin, B., 2008. Polysorbates 20 and 80 used in the formulation of protein biotherapeutics: Structure and degradation pathways. J. Pharm. Sci. 97(8): 2924-2935.

Kiese, S., Pappenberger, A., Friess, W., Mahler, H-C.,. 2008. Shaken, not stirred: Mechanical stress testing of an IgG1 antibody. J. Pharm. Sci. 97(10): 4347-4366.

Kim, A., Akers, M., Nail, S., 1998. The physical state of mannitol after freeze-drying: Effects of mannitol concentration, freezing rate, and a noncrystallizing cosolute. J. Pharm. Sci. 87(8): 931-935.

Kosa, T., Maruyama, T., Otagiri, M., 1998. Species differences of serum albumins: II. Chemical and thermal stability. Pharm. Res. 15(3): 449-454.

Köhler, G., Milstein, C., 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256(5517): 495-497.

Labuza, T., Altunakar. B., 2007. Water activity prediction and moisture sorption isotherms. In: Barbosa-Cánovas, G., Fontana, A., Schmidt, S., Labuza, T. (Ed.): Water Activity in Foods - Fundamentals and Applications. Blackwell Publishing, Oxford, UK, pp. 109-154.

Lee, J., Timasheff, S., 1981. The stabilization of proteins by sucrose. J. Biol. Chem 256(14): 7193-7201.

Liu, W., Wang, D., Nail S., 2005. Freeze-drying of proteins from a sucrose-glycine excipient system: Effect of formulation composition on the initial recovery of protein activity. AAPS Pharm. Sci. Tech. 6(2): E150-157.

Lopes Jesus, A., Nunes, S., Ramos Silva, M., Matos Beja, A., Redinha, J., 2010. Erythritol: Crystal growth from the melt. Int. J. Pharm. 388: 129-135.

Luthra, S., Obert, J., Kalonia, D., Pikal M., 2007. Impact of critical process and formulation parameters affecting in-process stability of lactate dehydrogenase during the secondary drying stage of lyophilization: A mini freeze dryer study. J. Pharm. Sci. 96(9): 2242-2250.

Mahler, H-C., Müller, R., Friess, W., Delille, A., Matheus, S., 2005. Induction and analysis of aggregates in a liquid IgG1-antibody formulation. Eur. J. Pharm. Biopharm. 59: 407-417.

Mahler, H-C., Friess, W., Grauschopf, U., Kiese, S., 2009. Protein aggregation: Pathways, induction factors and analysis. J. Pharm. Sci. 98(9): 2909-2934.

Manning, M., Patel, K., Borchardt, R., 1989. Stability of protein pharmaceuticals. Pharm. Res. 6(11): 903-918.

Martins, S., Jongen, W., van Boekel, M., 2001. A review of Maillard reaction in food and implications to kinetic modelling. Trends Food Sci. Technol. 11(9-10): 364-373.

Moynihan, C., Easteal, A., Wilder, J., 1974. Dependence of the glass transition temperature on heating and cooling rate. J. Phys. Chem. 78(26): 2673-2677.

Muga, A., Arrondo, J., Sancho, B., Bernabeu, C., 1993. Structural and functional strudies on the interaction of sodium dodecyl sulfate with β-galactosidase. Arch. Biochem. Biophys. 300(1): 451-457.

Narhi, L., Schmit, J,. Bechtold-Peters, K., Sharma, D., 2012. Classification of protein aggregates. J. Pharm. Sci. 101(2): 493-498.

Nema, S., Avis, K., 1993. Freeze-thaw studies of a model protein, lactate dehydrogenase, in the presence of cryoprotectants. J. Parent. Sci. Tech. 47(2): 76-83.

Okamoto, N., Oguni, M., 1996. Discovery of crystal nucleation proceeding much below the glass transition temperature in a supercooled liquid. Solid State Commun. 99(1): 53-56.

Orford, P., Parker, R., Ring, S., 1990. Aspects of the glass transition behavior of mixtures of carbohydrates of low molecular weight. Carbohydr. Res. 196: 11-18.

Paborji, M., Pochopin, N., Coppola, W., Bogardus, J., 1994. Chemical and physical stability of chimeric L6, a mouse-human monoclonal antibody. Pharm. Res. 11(5): 764-771.

Pace, C., 1975. The stability of globular proteins. Crit. Rev. Biochem. Mol. Biol. 3(1): 1-43.

Palumbo, M., Smith, P., Strange, E., van Hekken, D., Tunick, M., Holsinger, V., 1995. Stability of β-Galactosidase from *Aspergillus oryzae* and *Khyveromyces lactis* in dry milk powders. J. Food Sci. 60(1): 117-119.

Piedmonte, D., Summers, C., McAuley, A., Karamujic, L., Ratnaswamy, G., 2007. Sorbitol crystallization can lead to protein aggregation in frozen protein formulations. Pharm. Res. 24(1): 136-146.

Pikal-Cleland, K., Carpenter, J., 2001. Lyophilization-induced protein denaturation in phosphate buffer systems: Monomeric and tetrameric β-galactosidase. J. Pharm. Sci. 90(9): 1255-1268.

Pikal, M., Shah, S., Roy, M., Putman, R., 1990. The secondary drying stage of freeze drying: Drying kinetics as a function of temperature and chamber pressure. Int. J. Pharm. 60(3): 203-217.

Prestrelski, S., Tedeschi, N., Arakawa, T., Carpenter, J., 1993a. Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. Biophys. J. 65(2): 661-671.

Prestrelski, S., Arakawa, T., Carpenter, J., 1993b. Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress-specific stabilization. II. Structural studies using infrared spectroscopy. Arch. Biochem. Biophys. 303(2): 465-473.

Pyne, A., Chatterjee, K., Suryanarayanan, R., 2003. Crystalline to amorphous transition of disodium hydrogen phosphate during primary drying. Pharm. Res. 20(5): 802-803.

Randolph, T., Carpenter, J., 2007. Engineering challenges of protein formulations. AIChE J. 53(8): 1902-1907.

Reid, D., 2007. Water activity: Fundamentals and relationships. In: Barbosa-Cánovas, G., Fontana, A., Schmidt, S., Labuza, T. (Ed.): Water Activity in Foods - Fundamentals and Applications. Blackwell Publishing, Oxford, UK, 15-28.

Remmele, R., Nightlinger, N., Srinivasan, S., Gombotz, W., 1998. Interleukin-1 receptor (IL-1R) liquid formulation development using differential scanning calorimetry. Pharm. Res. 15(2): 200-208.

Rodriguez Furlan, L., Lecot, J., Perez Padilla, A., Campderros, M., Zaritzky, N., 2011. Effect of saccharides on glass transition temperatures of frozen and freeze dried bovine plasma protein. J. Food Eng. 106(1): 74-79.

Roos, Y., 1993. Melting and glass transitions of low molecular weight carbohydrates. Carbohydr. Res. 238: 39-48.

Roos, Y., 1997. Frozen state transitions in relation to freeze drying. J. Therm. Anal. 48(3): 535-544.

Rosenberg, A., 2006. Effects of proteins aggregates: An immunologic perspective. AAPS J. 8(3): E501-E507.

Sambrook, J., Fritsch, E., Maniatis, T., 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, New York, USA.

Sarciaux, J-M., Mansour, S., Hageman, M., Nail, S., 1999. Effects of buffer composition and processing conditions on aggregation of bovine IgG during freeze-drying. J. Pharm. Sci. 88(12): 1354-1361.

Schersch, K., Betz, O., Garidel, P., Muehlau, S., Bassarab, S., Winter, G., 2010. Systematic investigation of the effect of lyophilizate collapse on pharmaceutically relevant proteins I: Stability after freeze-drying. J. Pharm. Sci. 99(5): 2256-2278.

Schersch, K., Betz, O., Garidel, P., Muehlau, S., Bassarab, S., Winter, G., 2012. Systematic investigation of the effect of lyophilizate collapse on pharmaceutically relevant proteins, part 2: Stability during storage at elevated temperatures. J. Pharm. Sci. 101(7): 2288-2306.

Schwendeman, S., Costantino, H., Gupta, R., Siber, G., Klibanov, A., Langer, R., 1995. Stabilization of tetanus and diphtheria toxoids against moisture-induced aggregation. Proc. Natl. Acad. Sci. USA 92(24): 11234-11238.

Searles, J., Carpenter, J., Randolph, T., 2001. Annealing to optimize the primary drying rate, reduce freezing-induced drying rate heterogeneity, and determine Tg' in pharmaceutical lyophilization. J. Pharm. Sci. 90(7): 872-887.

Shalaev, E., Franks, E., 1995. Structural glass transitions and thermophysical processes in amorphous carbohydrates and their supersaturated solutions. J. Chem. Soc. Faraday Trans. 91(10): 1511-1517.

Shalaev, E., Franks, F., 1996. Changes in the physical state of model mixtures during freezing and drying: Impact on product quality. Cryobiology 33: 14-26.

Shire, S., Shahrokh, Z., Liu, J., 2004. Challenges in the development of high protein concentration formulations. J. Pharm. Sci. 93(6): 1390-1402.

Singh, S., Afonina, N., Awwad, M., Bechtold-Peters, K., Blue, J., Chou, D., Cromwell, M., Krause, H-J., Mahler, H-C., Meyer, B., Narhi, L., Nesta, D., Spitznagel, T., 2010. An industrial perspective on the monitoring of subvisible particles as a quality attribute for protein therapeutics. J. Pharm. Sci. 99(8): 3302-3321.

Sominanda, U., Rot, U., Suoniemi, M., Deisenhammer, F., Hillert, J., Fogdell-Hahn, A., 2007. Interferon beta preparations for the treatment of multiple sclerosis patients differ in neutralizing antibody seroprevalence and immunogenicity. Mult. Schler. 13: 208-214.

Surewicz, W., Mantsch, H., Chapman, D., 1993. Determination of protein secondary structure by Fourier transform infrared spectroscopy: A critical assessment. Biochem. 32(2): 389-394.

Tanaka, K., Takeda, T., Miyajima, K., 1991. Cryoprotective effect of saccharides on denaturation of catalase by freeze-drying. Chem. Pharm. Bull. 39(5): 1091-1094.

Tang, X., Pikal. M., 2004. Design of freeze-drying processes for pharmaceuticals: Practical advice. Pharm. Res. 21(2): 191-200.

Taylor, L., Zografi. G., 1998. Sugar-polymer hydrogen bond interactions in lyophilized amorphous mixtures. J. Pharm. Sci. 87(12): 1615-1621.

Thirumangalathu, R., Krishnan, S., Speed Ricci, M., Brems, D.,, Randolph, T., Carpenter, J., 2009. Silicone oil- and agitation-induced aggregation of a monoclonal antibody in aqueous solution. J. Pharm. Sci. 98(9): 3167-3181.

Timasheff, S., 1992. Water as ligand: Preferential binding and exclusion of denaturants in protein unfolding. Biochem. 31(41): 9857-9864.

Timasheff, S., 1998. Control of protein stability and reactions by weakly interacting cosolvents: The simplicity of the complicated. Adv. Protein Chem. 51: 355-432.

Tyagi, A., Randolph, T., Dong, A., Maloney, K., Hitscherich, C., Carpenter, J., 2009. IgG particle formation during filling pump operation: A case study of hetereogeneous nucleation on stainless steel nanoparticles. J. Pharm. Sci. 98(1): 94-104.

Usami, A., Ohtsu, A., Takahama, S., Fujii, T., 1996. The effect of pH, hydrogen peroxide and temperature on the stability of human monoclonal antibody. J. Pharm. Biomed. Anal. 14: 1133-1140.

Varshney, D.., Kumar, S., Shalaev, E., Kang, S-W., Gatlin, L., Suryanarayanan, R., 2006. Solute crystallization in frozen systems - Use of synchrotron radiation to improve sensitivity. Pharm. Res. 23(10): 2368-2374.

Vasiljevic, T., Jelen, P., 2003. Drying and storage of crude β-galactosidase extracts from *Lactobacillus delbrueckii sp. bulgaricus 11842*. Innov. Food Sci. Emerg. Tech. 4(3): 319-329.

Vazquez-Rey, M., Lang, D., 2011. Aggregates in monoclonal antibody manufacturing processes. Biotechnol. Bioeng. 108(7): 1494-1508.

Vermeer, A., Bremer, M., Norde, W., 1998. Structural changes of IgG induced by heat treatment and by adsorption onto a hydrophobic Teflon surface studied by circular dichroism spectroscopy. Biochim. Biophys. Acta 1425: 1-12.

Vlieghe, P., Lisowski, V., Martinez, J., Khrestchatisky, M., 2010. Synthetic therapeutic peptides: Science and market. Drug Discov. Today 15: 40-56.

Wang, B., Tchessalov, S., Warne, N., Pikal, M., 2009. Impact of sucrose level on storage stability of proteins in freeze-dried solids: I. Correlation of protein-sugar interaction with native structure preservation. J. Pharm. Sci. 98(9): 3131-3144.

Wang, W., 1999. Instability, stabilization, and formulation of liquid protein pharmaceuticals. Int. J. Pharm. 185(2): 129-188.

Wang, W., 2000. Lyophilization and development of solid protein pharmaceuticals. Int. J. Pharm. 203(1-2): 1-60.

Wang, W., Nema, S., Teagarden, D., 2010. Protein aggregation - Pathways and influencing factors. Int. J. Pharm. 390(2): 89-99.

Wang, W., Singh, S., Zeng L., King, D., Nema, S., 2007. Antibody structure, instability, and formulation. J. Pharm. Sci. 96(1): 1-26.

Workman, E., Reynolds, S., 1950. Electrical phenomena occurring during the freezing of dilute aqueous solutions and their possible relationship to thunderstorm electricity. Phys. Rev. 78(3): 254-260.

Yoshioka, S., Aso, Y., 2005. A quantitative assessment of the significance of molecular mobility as a determinant for the stability of lyophilized insulin formulations. Pharm. Res. 22(8): 1358-1364.

Yoshioka, S., Miyazaki, T., Aso, Y., Kawanishi, T., 2007. Significance of local mobility in aggregation of β-galactosidase lyophilized with trehalose, sucrose or stachyose. Pharm. Res. 24(9): 1660-1667.

Yutani, K., Ogasahara, K., Tsujita, T., Sugino, Y., 1987. Dependence of conformational stability on hydrophobicity of the amino acid residue in a series of variant proteins substituted at a unique position of tryptophan synthase alpha subunit. Proc Natl Acad Sci USA 84(13): 4441-4444.

Zhang, J., Zografi. G., 2000. The relationship between "BET"- and "free volume"-derived parameters for water vapor absorption into amorphous solids. J. Pharm. Sci. 89(8): 1063-1072.